UNIVERSITÀ
DEGLI STUDI
DI TORINO

Università degli Studi di Torino

Department of Veterinary Sciences

PhD in VETERINARY SCIENCES FOR ANIMAL HEALTH
AND FOOD SAFETY

XXXIV cycle

INTEGRATED APPROACH TO THE EVALUATION OF THE WELFARE AND MANAGEMENT IN THE EQUINE MEAT FARM

PhD candidate: Dr. Federica Raspa

Supervisor: Prof. Emanuela Valle

PhD Coordinator: Prof. Maria Teresa Capucchio

Scientific Disciplinary Sector: AGR18 – Animal Nutrition and Feeding

Academic years

2018-2021

SUMMARY

Not enough effort is being made to safeguard the welfare of horses reared for meat production. Moreover, there is a lack of scientific knowledge concerning the welfare of horses reared in this way. These horses are often kept in intensive breeding farms where they are housed in group pens at high stock densities and fed high starch diet. The present PhD project aimed to apply an integrated approach to the evaluation of the welfare and management in the equine meat farm. The integrated approach was developed considering several aspects – welfare indicators, gut health, behaviour, production performances – which were investigated according to two main aims.

The first aim was related to the welfare assessment of horses reared for meat production – to obtain insight into their housing and management welfare conditions and evaluate the selected welfare indicators and behavioural activities were influenced by the main causes of concern that regard intensive breeding farms: stocking density and feeding management.

Considering the welfare indicators, a checklist adapted from the Animal Welfare Indicators (AWIN) assessment protocol was developed and employed to assess whether welfare indicators were influenced by stocking densities (m^2/horse) and feeding strategies applied. An analysis was carried out on the data obtained from 7 surveys conducted at a single horse farm designed for meat production. In each survey, the same 12 pens were assessed, but on each occasion, the horses in the pens had been changed as had the stocking densities. Briefly, 561 horses aged 16 ± 8 months (mean ± standard deviation, SD) were evaluated. Two stocking density cut-off values (median and 75th percentile: 3.95 and 4.75 m^2/horse, respectively) were applied to investigate the effect of stocking density on horse welfare. Data were analysed using Mann–Whitney U and Fisher's exact tests (p<0.05). When cut-off was set as the median percentile, lower stocking density was associated with improvements in body condition score (BCS), coat cleanliness and bedding quantity, less coughing, less resting in a standing position, and less feeding related to the greater space available at the feed bunk. When the 75th percentile cut-off was used, indicators that improved were coat cleanliness, bedding quantity and mane and tail condition, as well as less resting in standing position and less feeding related to the greater space available at the feed bunk. Further increment of space and/or changes in management regimes should be investigated to improve all the indicators. Moreover, results related to feeding indicated the need to intervene as starch intakes exceeded recommended safe levels, negatively affecting horse welfare.

Considering behavioural activities, an ethogram of 13 mutually exclusive behavioural activities was developed. Behavioural observations were performed over a 72 h period on group pens selected on the basis of stocking density and the homogeneity of breed, age, height at the withers, and time since arriving at the farm. Scan sampling (n=96 scans/horse/day) was used on 22 horses. The mean frequency (%) ± SD for each behavioural activity was calculated to obtain the time-budget. The associations between time-budget and stocking density were evaluated using a bivariate analysis. The relationships were analysed by Pearson's correlation coefficient (r). Data revealed an unusual time-

budget compared to that of wild-living horses, where the main behavioural activity expressed was standing (30.56% ± 6.56%), followed by feeding (30.55% ± 3.59%), lying (27.33% ± 2.05%), and locomotion (4.07% ± 1.06%). Moreover, the results obtained showed that locomotion, playing, and self-grooming positively correlated with a reduction in stocking density, indicating the potential to use these behaviours as positive welfare indicators for young horses kept in group pens. Therefore, the reduction in stocking density and as a consequence a space allowance of 6 m^2/horse had a positive impact on the expression of locomotion, playing, and self-grooming which could be proposed as indicators of positive welfare in young horses kept in group pens.

The second aim of the present PhD project was related to the feeding management – to evaluate the effects of two feeding managements (one based on high amounts of starch vs. one based on high amounts of fibre) on gut health, behaviour and production performances. Nineteen Bardigiano horses (12 females and 7 stallions), 14.3 ± 0.7 months of age, were randomly assigned to two groups — one fed with high amounts of starch (HS; n=9; 43% hay plus 57% cereal grain-based pelleted feed) vs. one fed with high amounts of fibre (HF; n=10; 70% hay plus 30% pelleted fibrous feed).

Considering gut health, after horses were slaughtered, stomachs were scored for gastric mucosa lesions. Samples of gut content and gut wall were taken from different intestinal compartments of the horse digestive tract. Gut content from each intestinal compartment was analysed for dry matter, organic matter, ash content, particle size distribution and volatile fatty acid composition. Gut wall from each selected intestinal compartment was evaluated for morphometric and histopathological indices. Moreover, mesenteric lymph nodes and liver samples were collected to evaluate their microbiological contamination. Data were analysed by linear mixed-effects model in which dietary treatment, sex and their interaction were set as the model's fixed effects. Each horse within each sex and diet group was considered an experimental unit and used as the random variable for all analyses. The glandular region of horses in HS group presented gastric mucosa lesions significantly more severe compared to those seen in horses belonging to the HF group (p=0.01). Moreover, horses fed HS diet presented a higher dry matter content (p<0.01) in the right dorsal colon, a higher organic matter (p<0.01) and a higher ash content (p<0.01) in the sternal flexure, pelvic flexure, right dorsal colon and rectum. In these latter intestinal compartments, horses fed a HS diet also showed a higher proportion of particles retained on an 8 mm sieve (p<0.05) and a higher proportion of particles that washed through the finest sieve (<1 mm) (p<0.05). Moreover, the total amounts of volatile fatty acids as well as valeric acid were found to be significantly higher in horses fed the HS vs. HF diet (p<0.01). Interestingly, valeric acid was increased in horses receiving the HS diet, and this should be explored in more depth since this VFA has already been implicated in causing alterations to the gastric mucosa. In fact, the HS diet was associated with the presence of more severe mucosa gastric lesions in the glandular region of the stomach (p=0.01) and a higher lymphoplasmacytic inflammation in the jejunum (p=0.01) and pelvic flexure (p=0.05); instead no differences were found regarding the histo-morphometry of duodenum, jejunum

and ileum compared to the HF diet. Moreover, the results showed an increased intestinal permeability in the horses fed HS compared to HF, according to the significant increased total mesophilic aerobic bacteria counts in mesenteric lymph nodes (p=0.04) and liver samples (p=0.05). In summary, the results of this study confirm that the diet composition, and thus feeding management practices, are able to influence the gut environment and its functioning.

Considering behavioural activities, during the feeding trial, one 2D camera was installed on each pen. The ethogram previously developed and published based on 13 mutually exclusive behavioural activities was used. Behavioural observations were carried out over a 96 h period by using scan sampling (n=144 scans/horse/day for a total of 10,368 scans sampled). The mean frequency (%) for each behavioural activity was calculated and behavioural data were checked for normality, employing the Shapiro–Wilk test. One-way ANOVA or Wilcoxon test were used to analysed data according to their distribution. The significance level was set at p>0.05. The results showed that the behavioural changes generated by feeding horses with a fibre-based diet indicated an increased welfare, according to the increased expression of the feeding behaviour and the reduced frequencies of standing and locomotion in horse fed HF diet compared to the horses fed the HS diet. Moreover, feeding horses with the fibre-based diet resulted in a lower expression of stereotypic behaviour and biting compared with horses fed with a HS diet. In summary, the change in feeding management from a HS diet to a HF diet in horses reared for meat production led to advantage on the horse's welfare since horses fed the HF diet showed less aggressive and stereotypic behaviours as well as on the economic point view since horses fed the HF diet were less engaged in by locomotion – so, spending less energy – and more occupied in feeding behaviour.

Considering production performances, at slaughter on abattoir, biological and tissue samples were collected to evaluate the oxidative status of the horse. Moreover, meat quality traits – chemical composition and fatty acid profile of the *Longissimus thoracis et lumborum* muscle – were evaluated. A linear mixed model was used: dietary treatment and sex were fixed effects and their interaction analysed on production and metabolic parameters as dependent variables. HS diet did not result with any difference in daily bodyweight gain or with any positive effect on meat quality traits. Horses in HS showed increased muscle pH (p=0.02), lighter muscle colour (L) (p=0.01), lower muscle protein content (p=0.01), increased intramuscular fat concentrations (p=0.03) but lower concentration of muscle PUFAs (p=0.05). The HS diet increased muscle glutathione peroxidase and superoxide dismutase activities (p=0.01 and p=0.03, respectively). Moreover, horses in HS had, and lower plasma catalase activities (p=0.05), suggesting that those animals were less protected by oxidative damages. Therefore, the HS diet resulted wasteful for an economic point of view since it did not result in any increase in daily bodyweight gain or with any positive effect on meat quality traits. Moreover, the results obtained showed that feeding horses HS diet can have global effects on horse physiology, and thus represents a threat for their welfare.

INDEX

LIST OF ABBREVIATIONS

ADF: acid detergent fibre

ADL: acid detergent lignin

AWIN: Animal Welfare Indicators

BCS: body condition score

BW: bodyweight

CAE: apex of the caecum

CAT: catalase

Cd: crypt depth

DM: dry matter

DNPH: dinitrophenylhydrazine

DU: duodenum

ESGD: Equine Squamous Gastric Disease

EGGD: Equine Glandular Gastric Disease

FAME: fatty acid methyl esters

GPx: glutathione peroxidase

HCl: hydrochloric acid

HF: high fibre

HPLC: High Performance Liquid Chromatography

HS: high starch

HSD: high stocking density

HY: lipid hydroperoxides

ILE: ileum

JEJ: jejunum

LSD: low stocking density

MADC: digestible crude protein

MUFA: monounsaturated fatty acids

NDF: neutral detergent fibre

NSC: non-structural carbohydrates

OM: organic matter

PF: pelvic flexure

PUFA: polyunsaturated fatty acids

RDC: right dorsal colon

RE: rectum

SI: small intestine

SF: sternal flexure

SFA: saturated fatty acids

SOD: superoxide dismutase

TBARS: thiobarbituric acid reactive substances

UFA: unsaturated fatty acids

VFAs: volatile fatty acids

Vh: villus height

Vh/Cd: villus height to crypt depth ratio

Vw: villus width

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

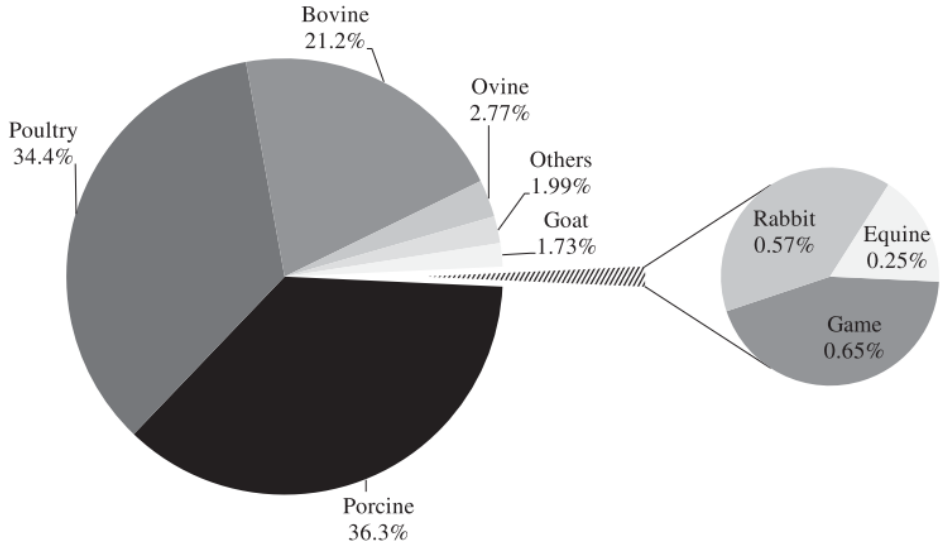
The domesticated horses have deeply influenced the mankind. Horses have been used for a variety of purposes including for meat as a means of agricultural activities, transport and for leisure. This variability still exists today and depends of the type of horse and where it is to be found in the world [1]. It is reported that the horse was possibly first domesticated – at the end of the Neolithic era, around 5000-6000 B.C. – primarily for meat [2]. Figure 1 represents one of the 600 parietal wall paintings that were found in Lascaux Cave in southwestern France – UNESCO World Heritage List from 1979. The paintings represent the typical local fauna that correspond with the fossil record of the Upper Paleolithic in the area. In particular, the age of the painting is estimated at around 17,000 years. The present wall painting indicates that since the ancient time, horses were hunted to provide meat for human consumption.

Figure 1. Cave painting of a horse at Lascaux.
(Source: https://it.m.wikipedia.org/wiki/File:Lascaux,_horse.JPG)

1.1. The horse meat production

The horse meat consumption depends on cultural and traditional customs; thus the horse meat can be considered as a niche product. In fact, it is reported that — by comparison to other meat-producing species like pork, poultry, bovine or ovine — horse meat represents only 0.25% of the total worldwide meat production (Figure 2) [3]. Considering the European community countries, Faostat data [4] shows that more than 500,000 horses are slaughtered in Europe each year to produce meat destined to human consumption. In particular, Spain stands out as the major horse meat producer (17%), followed by Italy (16%), Romania (14%), Poland (11%), and France (8.2%) [3,5].

Figure 2. Worldwide meat production (%) by different meat-producing species. Adapted from Belaunzaran et al., 2015 [3].



Interestingly, reviewing the scientific literature, it seems that most of the available scientific studies are focused on the final product — the meat — in terms of its consumption [3] and nutritional values [5,6]. The nutritional value of the horse meat is related to the high bioavailable iron content (3.89 mg/100 g) which is almost twice that of the other red meat. Moreover, horse meat is characterised by low intramuscular fat and low cholesterol concentration [7]. In particular, it is characterised by a more significant proportions of unsaturated fatty acids compared to other red meat types [8,9]. However, it is important to consider that several factors can influence the fatty acids profile of the horse meat as the production systems, the slaughter age, the sex of the animals and the feeding management adopted [10]. However, little knowledge is available on the housing and management welfare conditions of horses reared for meat purpose. It is reported that there are no standardised farming conditions for the breeding of these animals [5]. Moreover, the present breeding system includes several concerns: the absence of adequate traceability, the low horsemeat consumption, the high number of horses imported from the East European countries – unfortunately slaughtered often illegally – the lack of specific production guidelines [5].

What it is clear from the existing scientific literature is that in the past, most horse meat derived from the slaughter of horses at the end of their working lives, whereas, nowadays, horse meat is mainly obtained from young horses which are fattened for meat purpose [3,7]. In particular, these animals seem to be often kept in intensive farming systems characterised by overcrowding and intensive feeding management in order to reduce the length of the fattening period and to increase the meat production performances [5].

1.2. The welfare assessment of the horse

Although the farming of horses reared for meat production is an existing reality, there is a lack of scientific studies as well as welfare guidelines and/or regulations assessing equine faming conditions and how to safeguard horse welfare at farming level [11]. When we talk about welfare assessment, we refer on a complex task that requires a multidimensional approach that involves the examination of a panel of welfare indicators encompassing all components of animal welfare [12]. It is possible to recognise three categories of indicators: resource-based, management-based, and animal-based [13,14]. Some criticisms have been made regarding the application of protocols built on animal-based indicators due to the difficulty in applying them at the farm level – the protocols being very time-consuming and costly [15]. However, today the only document validated at European Union level for the welfare assessment of equines is represented by the Animal Welfare Indicators (AWIN) welfare assessment protocol for horses and for donkeys [16,17].

The AWIN protocol is based on the assessment of animal-based indicators and follows the Welfare Quality® approach that consists of four welfare principles and twelve welfare criteria [18]. The four welfare principles are good feeding, good housing, good health, and appropriate behaviour. They represent the founding elements of the Five Freedoms [19] since they describe the needs of animals that should be satisfied in order to cover all aspects of animal welfare [18]. However, some limitations of the AWIN protocol for assessing horse's welfare can be underlined.

Firstly, it is needed to remember that the AWIN protocol was developed in relation to horses aged 5 years or older. Therefore, it is not adequate for the welfare assessment of horses younger than 5 years old as the horses that are reported to be reared for meat production [7]. Moreover, the welfare principles not always include welfare indicators that fit the description of the needs of the animals. As already stated for dairy donkeys in a previous published paper [20], the AWIN protocol needs to be implemented with other requirements and indicators that should be taken into account to properly assess the welfare of equines.

1.3. Good feeding

Among the welfare principles, good feeding plays a crucial role for equines [20]. However, at the moment, the welfare principle of good feeding is described by two different welfare criteria: the appropriate nutrition and the absence of prolonged thirst. In particular, the Body Condition Score (BCS) represents the only welfare indicator considered in the AWIN protocol to measure the welfare criteria of appropriate nutrition for equines. However, the BCS aims to assess the body fat reserves through the visual appraisal and palpation of specific anatomic key areas [21]. Although its subjective nature, it is reported that it can be a useful tool when it is performed according to specific protocol [22]. However, it is needed to keep in mind that it is not an indicator of the quality of the diet supplied – e.g.

the forage:concentrate ratio, the stage of maturity of the forage, the hygienic quality of the feeds and the ethological need of foraging of horses.

The starting point to properly manage the horse and so to safeguard its welfare is to know who the horse is. Accordingly, the feeding behaviour as well as the nutritional requirements of the horse are strongly linked to its gastrointestinal anatomy and physiology. Therefore, the following subsections include those aspects (the digestive system and the food pyramid of the horse) that should be considered in respecting the best practices for the feeding of horses.

1.3.1. The digestive system of the horse

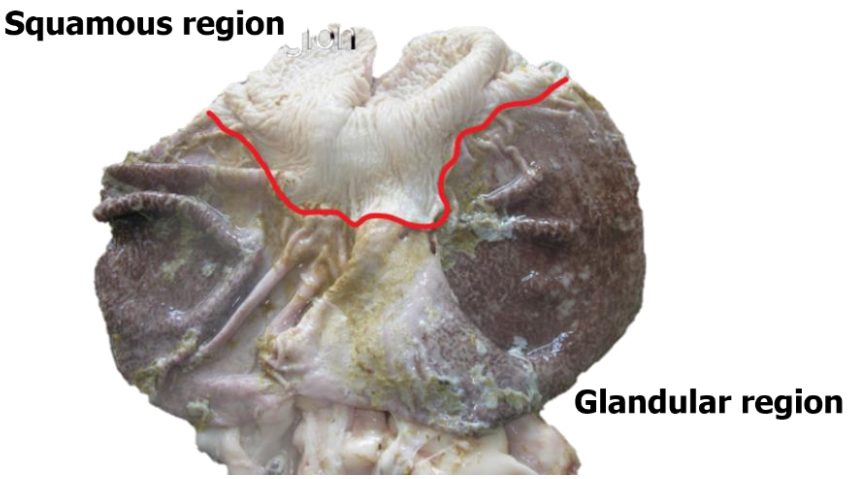
The horse is a grazing herbivore, monogastric and hindgut fermenter. The horse in nature spend most of its daytime in feeding behaviour and its digestive system is designed to receive small amounts of food in a continuous manner throughout the day – "tricke feeding" [23,24]. The horse has prehensile lips and the nature of the feeds fed influences its chewing rate and saliva production. Moreover, the horse does not salivate at the sight of food, but only while it is chewing [1]. It is reported that feeding 1 kg of hay results in 3 to 6 liters of saliva production, while feeding 1 kg of cereal grains results in 1 to 1.7 liters of saliva production in adult horses [25]. Saliva makes the bolus moister lubricating its passage to the stomach [1]. In the stomach it has a buffering function since the bicarbonate in saliva influences the stomach pH [26].

1.3.1.1. The stomach

The volume of the stomach represents only the 8.5% of the total gastrointestinal system; thus in an adult 500 kg horse contains around 8-15 liters of digesta. In nature, grazing promotes the presence of a protective "fibre mat" and a continuous flow of saliva that allow to buffer the stomach acid [27]. This condition avoids the harmful effects of the hydrochloric acid (HCl) on the gastric mucosa. In fact, the main secretory product of the glandular region of the stomach is the HCl that in horse is continuously secreted at a variable rate even when the stomach is empty [28]. For this reason, it is important to avoid prolonged fasting periods in stabled horses, otherwise the gastric acidity would damage the gastric mucosa. Due to the anatomical and physiological features of the stomach when wrong feeding plan and feeding management are used to feed horses, it is possible to have several health problems. Regarding the stomach, gastric ulceration presents the main incidence in horses [29]. According to the European College of Equine Internal Medicine Consensus Statement [30] gastric ulcers need to be descripted on the basis of their anatomical location. In fact, as shown Figure 3, the stomach of equines is characterised by two well-distinguishable regions: the squamous or non-glandular region and the glandular region, which are divided by the *margo plicatus.* The committee proposed to name Equine Squamous Gastric Disease (ESGD) as a term to describe erosive and ulcerative lesions in the squamous region of the stomach. Moreover, it proposed the term Equine Glandular Gastric Disease (EGGD) to

describe lesions found in the glandular region of the stomach. In particular, increased starch intake has been recognised as a nutritional risk factor for the onset of ESGD in both non exercising horses and animals working at various levels of intensity [29,30]. Instead, the prevalence of EGGD has been less understood and the majority of EGGD lesion were found in the pyloric antrum [30].

Figure 3. Horse's stomach opened by cutting along the great curvature. Squamous and glandular regions are separated by the margo plicatus (red line).



1.3.1.2. The small intestine

In equines, the small intestine is divided into duodenum, jejunum and ileum. Considering the body size of the horse, its small intestine is rather small – around 25 meters in length in the 500 kg adult; with duodenum of around 1 meter, ileum 0.7 meter, whereas the main length is represented by the jejunum. The mucosa of the wall of the small intestine is composed of finger-like villi, each of which is surrounded by a group of crypts. Enterocytes are connected to each other by tight junctions which represent the physical barrier that avoid the trans-mucosal flux of high-molecular weight substances such as bacteria from the gut lumen to the lymphatic and systemic circulation [31].

The bile duct and the primary pancreatic duct open into the duodenal diverticulum which is located around 15 cm aboral to the pyloric sphincter [28]. Liver and plasma release their secretions into duodenum.

As shown in Table 1, pancreatic amylase is produced in lower amounts compared to other animals. Whereas the equine pancreas secretes more lipase that is responsible for the digestion of fatty acids.

Table 1. Amylase and lipase concentration in the pancreatic tissue of several species. Values are expressed as Mean UI/mg of pancreatic protein. Adapted from Lorenzo-Figueras et al., 2007 [32] and Merrit and Julliand, 2013 [23].

Animal species	Amylase	Lipase
Adult horse	2.3	41.5
Adult pig	107	49
Adult rat	56	39
Calf 14 days –6 months	2.3	11

The amylase catalyses the carbohydrate hydrolysis – hydrolyzable carbohydrates include simple sugars, disaccharides and starch. The starch represents the main non-structural carbohydrates (NSC) as well as the main energy source of the grains. As a consequence of the limited production of amylase in comparison to other species, several authors has suggested to not feed horses with more than 2 grams of starch/kg BW/meal [24,33,34].

In addition, the small intestine represents the gastrointestinal area in which fat and protein digestion occur. Fat are primarily digested by the pancreatic lipase that is the main digestive enzyme of the pancreatic equine secretion (Table 1) [32]. Moreover, fatty acids are absorbed through the enterocytes thank to the function of the bile salts. Instead, the proteolytic activity is mainly performed in the ileum by endogenous enzymes and microorganisms [23].

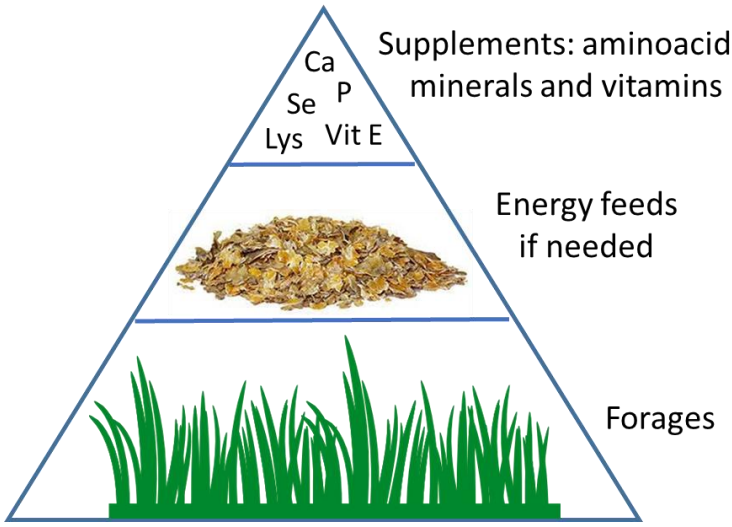
1.3.1.3. The large intestine

As hindgut fermenters, equines have a highly developed large intestine. In the adult 500 kg horse the mean caecal volume is 33 liters and that of the great colon is 80 liters [23]. Those volumes represent around the 60% of the total gastrointestinal volume of the horse. The hindgut shows an intense fibrolytic activity and the end-products of microbial fermentations are the volatile fatty acids (VFAs). VFAs are highly influenced by the diet. When horses are fed fibre-based diets in all segments the main VFAS produced are acetate (around 75% of the total), propionate (around 17% of the total) and butyrate (around 6% of the total) [23,35]; whereas little amounts of lactate are produced. On the contrary, with hay and concentrate rations, the percentage of acetate decreases in concert with an increase in the proportion of propionate and lactate [35]. Moreover, high amounts of starch-rich concentrate feeds alter the microbial ecosystem of the digestive tract of the horse and reduces the fibre digestion [36]. VFAs are the primary source of energy for horses. In fact, VFAs provide at least the 50-70% of the energy requirements of the horse [23] and they are quickly absorbed into the bloodstream and readily used as energy source or converted to glucose or fat [37].

1.3.2. The food pyramid of the horse

According to the nature of the horse, a fibre-based diet should represent the basis of the horse nutrition, respecting the innate herbivorous nature of this animal [1]. The fibre is provided by fresh or preserved forages supplied ideally ad libitum or offered in order to avoid more than 5 hours without foraging opportunity [38]. Therefore, forage represents the basis of the food pyramid of the horse (shown in Figure 4). In the middle of the food pyramid there are the energy-rich feeds as concentrate and oils which can be supplied when extra energy is needed to meet the nutritional requirements of the horse according to its physiological status (e.g. lactation, gestation, growing) or to the intensity of exercise/work. The top of the pyramid represents the supplements; thus aminoacids, vitaminis and minerals that are needed to adequately balance the diet according the physiological status and specific requirements of the horse.

Figure 4. The food pyramid diet of the horse.



1.3.2.1. Forages

The basis of horse's diet is the forage and, according to Harris et al., 2017 [38], it is needed to guarantee a minimum daily forage intake of 15 g DM/kg BW. This means that the minimum daily forage intake (considering a hay at a DM content of 85%) for an adult horse of 500 kg BW is 7.5 kg of hay (as DM basis) or 8.8 kg of hay (as fed basis).

Moreover, it is important to consider the quality of forage in terms of hygienic quality and stage of maturity. The hygienic quality of forages should be assessed at least with its visual and olfactory inspection [38]. The aim is to evaluate the presence of mould and dust that could predispose to the onset of important health problems affecting the respiratory tract and the gut.

The stage of maturity has an important effect on the energy and nutrient composition of the forage [39]. The energy content decreases with advancing maturity, but it is important to keep in mind that

the forage quality can be affected by the grass composition and climate changes [20]. Therefore, the nutrient analysis of forage is recommended above when horses have health disorders [38].

1.3.2.2. Energy feeds

If the forage is not sufficient to maintain the adequate body condition of the horse, extra energy should be introduced. On the market are available three different categories of energy-dense concentrates:
1) Traditional cereals-based concentrates: The main energy sources of these feedstuffs are starch and simple sugars. They are cereal grains concentrate feeds characterised by a mix of whole seeds (e.g. oat) and fakes (e.g. corn and/or barley flakes). Moreover, it is possible to find on the market cereal grains pelleted feeds. Generally, the raw materials are balanced with protein sources – e.g. alfalfa flour, soybean flour or fava beans (*Vicia faba*).
2) Fatted concentrates: labelled to contain an inclusion level of crude fat higher than 5%. Generally, they are sold as pelleted feeds. The percentage of crude fat varies between 6 to 8%, but some products reach higher inclusion rate of fats – for example when they are composed by raw materials as the rice bran.
3) Fat and fibre-based concentrates: labelled to contain an inclusion level of crude fibre around 17%. The energy sources of these feedstuffs are fat and superfibres. In particular, suprefibres are represented by beet pulps and soyban hulls.
Due to the demands placed on horses for competitions and/or productive performances (i.e. sport horses and horses reared for meat production), they are often fed with high amounts of cereals-based concentrate feeds rich in starch and simple sugars [33,40]. However, this feeding practice is associated with several health problems (see section 1.5). Some solutions include reducing the quantity of starch-rich concentrates and introducing forage-based products [37] that have a high energy content, since they contain some superfibres and fat.

1.4. Good housing

One important aspect that should be addressed in the AWIN protocol is related to the welfare principle of good housing in terms of space allowance per animals within group pen. As clearly underlined in the section dedicated to group housed horses, the AWIN protocol still needs to be refined and improved in light of the results of up-to-date scientific research. Therefore, further research should be carried out on horses kept in group pens as those reared for meat production. In fact, horses reared for meat production can be often kept in group pens with high stocking density. Stocking density is recognised as crucial to reaching an adequate level of welfare at farm level [41–44]. Accordingly, the general approach of the European Union to improve farm animal welfare is to increase the space allowance per animal within the group pens [45]. In fact, the minimum space requirements in group housing systems have been set for pigs [46], poultry [47], and cattle [48]. Instead, no specific European Union Directives

have been defined for meat production horses [11]. The first indications about minimum space requirements for horses housed in group pens have been provided by the Swiss Federal Council in the Animal Welfare Ordinance (TSchV) of 23 April 2008 [49]. In this document, the minimum space allowance per horse is based on the measurement of the height at the withers of the individual group members. This criterion was adopted in the AWIN protocol for horses [16]. However, according to Burla et al., 2017 [50], the minimal space requirements proposed by the TSchV and then by the AWIN are not based on scientific evidence and may not be adequate to guarantee adequate welfare for all horses of a given group [50].

The space allowance per horse within a group pen represents an important aspect that should be considered also in relation to the welfare principle of good feeding. In fact, equines need to receive an adequate diet to maintain body condition and the appropriate group size is important to avoid the risk of underfeeding or overfeeding of some animals [20]. The group size should be set on the basis of the space available; but also on the basis of the bunk length or feeding space. However, no mention is made in the AWIN protocol about this latter aspect. To the best knowledge, the only document that consider the feeding space per horse within a group is represented by the Canadian Code of Practice for the Care and Handling of Equines [51]. This document recommends guaranteeing at least 1 meter feeding space per horse under group-housing conditions and suggests having an extra feeding point available (i.e., one feeding point more than the number of horses).

Avoid overcrowding within the group pen is crucial to guarantee sufficient space for all horses in order to allow them to express their natural lying and moving behaviour and to reduce competition for available resources [52]. What it is clear is that high-density group housing can negatively affect animal welfare. Benhajali et al., 2008 [53] described the social behaviours of 44 densely housed mares kept in a paddock and found a reduction in the expression of social behaviours replacing by agonistic behaviours with a total absence of positive social interactions (e.g. mutual grooming) and lying and rolling. Also other authors studied the effects of the space allowance on horse's behaviours [54]; but to the best knowledge studies have evaluated the effects of space allowance on the welfare indicators of horses kept in group pens. Moreover, no studies have evaluated to date whether an increase in the space allowance per horse kept in a group pen can generate an improvement in behavioural indicators which may express a condition on increased welfare.

1.5. Good heath

The welfare principle of good health is described by three different welfare criteria: the absence of injuries, the absence of disease, the absence of pain and pain induced by management procedures. The AWIN protocol evaluate these present welfare criteria by a panel of welfare indicators that are listed in the section dedicated to group housed horses. However, as stated in the AWIN protocol, those welfare indicators should be improved and refined according to new researches. Therefore, new

proposals for the assessment of welfare principle of good health were performed during the present PhD project according to the development of a specific checklist for the welfare assessment of horses reared for meat production and kept in group pen.

However, it is needed to underline the importance of the concept of gut health because it is often considered as a synonymous of animal health [55]. In fact, the concept of gut health is becoming progressively more important in the field of the animal nutrition. In human medicine gut health is often defined as "absence of clinical diseases; whereas this definition cannot be applied in animals [56]. According to Celi et al., 2019 [57], gut health is a multidimensional concept that depends on the maintenance of a delicate balance between the host, the gut microbiota, the structure and the function of intestinal barrier and the dietary compounds. Accordingly, diet is recognised one of the most important factors in influencing the gut health of the animals [58].

The gut microbiota is composed by viruses, archaea and bacteria and it benefits the host by modulating the development and function of the immune system [59,60] and by playing an important role in digestive function – providing nutrients from dietary substrates [38]. The gut microbiota is also known to play a role in the gut-brain axis and behaviour through the release of microbial metabolites – e.g. volatile fatty acids, neurotransmitters and catecholamines – that cross the blood-brain barrier once they are absorbed by the intestinal epithelium and released into the bloodstream [61]. Interestingly, changes in gut microbiota composition has been associated with the increased expression of anxiety-like behaviours in horses [61]. Gut microbiota and its interaction with the intestinal barrier function has been mainly studied in poultry [62] and pigs [63]. It was found that gut microbiota alterations can affect the intestinal histo-morphology through the modifications of villus height and crypt depth. In particular, villus height and crypt depth have been proposed as indicators of gut health and functionality [64]. Ideally, the intestinal barrier should be characterised by long villi and shallow crypts. In fact, long villi are associated with an adequate mucosal absorptive area, whereas shallow crypts reflect the prolonged survival of villi [65]. Little studies on intestinal histo-morphology has been carried out in horses. Wambacq et al., 2020 [66] studied the effects of a dietary supplementation of sodium butyrate in healthy horses on – among the others –villus length and crypt depth of duodenum, jejunum and ileum. The authors did not find any effect on the histo-morphometry of the small intestine of the horses involved in their study and suggested that findings in poultry or pigs may not be directly translated to equines. However, they involved only fourteen adult warmblood horses and, actually, no similar studies seem to be present in the scientific literature. Therefore, it is really difficult to make any comparison.

Moreover, it is reported that one of the main cause of alterations of the gut microbiota in horses is related to feed starch-rich diet [31]. Several studies have underlined that feeding horses high amounts of starch constitutes a risk factor for the onset of gastrointestinal disorders such as gastric ulcers and colic [24,26], metabolic disorders such as acidosis and laminitis [67–69], and may cause changes in the time-budget or behavioural repertoire of the horse [70–72]. Accordingly, the safe level of 2 grams

of starch/kg BW/meal has been set by several authors [24,33,34]. This safe level is related to the limited ability of the horse to digest high amounts of starch as a consequence of the limited production of α-amylase in comparison to other species (see Table 1) [23,32]. Even though this recommendation, it is reported that often the feeding managements of the horses – riding or leisure horses as well as horses reared for meat production – are characterised by high amounts of concentrates as high starch cereal grains [73–75].

This feeding management causes a poorly buffered and acid environment in the stomach increasing the risk for the onset of gastric ulcers. Moreover, when the indigested starch reaches the hindgut, it causes alterations in the gut environment, leading to an increase in lactic acid production and a drop in pH with subsequent acidosis [23,34]. This condition increases the risk for colic and diarrhea [24,26]. It is stated that intestinal acidosis causes severe damage to the intestinal epithelium, leading to a condition of hyperpermeability – also known as 'leaky gut' [31]. Alterations in intestinal permeability can also lead to the translocation of enteric bacteria and/or their products from the gut lumen into the mesenteric lymph nodes and the portal circulation [31,76], with the potential for systemic consequences. Therefore, both diet composition and feeding management are able to influence gut barrier and gut function – i.e. the absorption and digestion of nutrients – by causing alterations to the gut environment in terms of its microbial profile, volatile fatty acids and the particle size distribution [36,77].

As stated before, in a 500 kg adult horses, the digestive system presents a length and volume of more than 30 meters and 150 liters, respectively. Therefore, another aspect that should be taken into account is that each intestinal compartment hosts a microbiota with a specific composition and there is also a lack of information regarding the differences between the luminal and the mucosal microbiota throughout the equine gut [78]. Accordingly, the utility of faecal samples as representative of the gut microbiota is problematic [60]. However, most studies have used faecal samples for their analyses, being easy and non-invasive to collect, meaning that direct evidence on the differential effects of diet on the distinct intestinal compartments remains sparse [79].

1.6. Appropriate behaviour

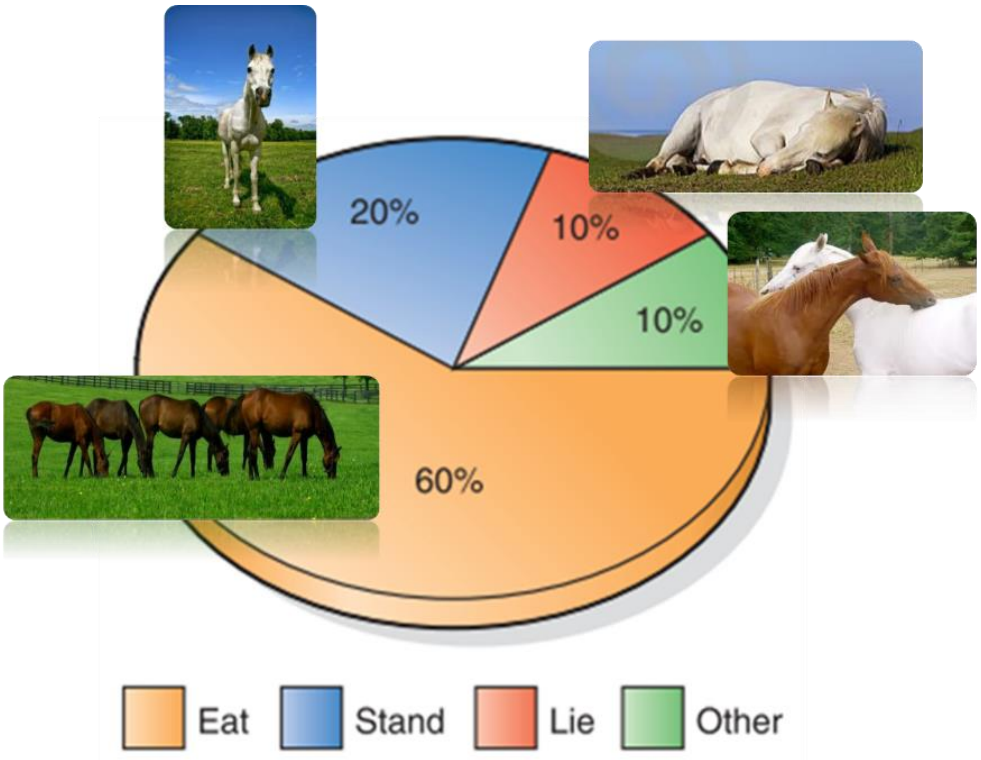
The study of animal behaviour is important to obtain insight into the welfare of the animals. According to the three dimensional-concept proposed by Fraser et al., 1997 [80], which integrates the Five Freedoms [19], an animal welfare assessment needs to encompass the study of animal behaviour. The natural-living orientation proposed by Fraser et al., 1997 [80], underline the importance for animals to have the possibility to live a relatively natural life, thus to express behaviours closer as possible to those performed in nature. Accordingly, studying the behaviours of animals reared in human-managed environments and comparing their time-budgets with those of animals living in natural environments is important for understanding animal welfare in the former [81,82]. This consideration results particularly

important when we talk about horses. In fact, despite the process of domestication, horses have maintained the species-specific behaviours of their wild ancestors [83].

Under natural living conditions feral and domestic pasture horses graze and browse for at least 60% equal to 16-18 hours of the daytime, spend only 3-4 hours on non-foraging behaviours and fasting not exceed 4 hours [1]. As shown in Figure 5, in nature the main behavioural activity expressed by the horse is grazing while freely and slowly moving. Accordingly, the horse is defined a trickle feeder. The constant movement during the grazing activity can be considered strictly linked to the feeding behaviour of the horse. The second behaviour more expressed during the day for the 20% of the daytime is standing; followed by lying (10%) and by other behaviours (10%) as social behaviours – e.g. mutual-grooming, playing and so on.

Since the horse in nature spend most of its daytime in feeding behaviour, it is clear that the feeding management of the stabled horses is crucial to safeguard their welfare. However, nowadays horses are often confined to single boxes or in group pens – so with little possibilities to freely move during the day – and fed just two or three daily feed rations, leading to unnatural long fasting times [84].

Figure 5. Behaviours expressed by horse in nature – frequency (%) of time during the day. Adapted from McGreevy, 2004 [85].



2. AIMS OF THE PROJECT

According to the title of the present PhD project, it was applied an integrated approach to the evaluation of the welfare and management in the equine meat farm. The integrated approach was developed considering several aspects – welfare indicators, gut health, behaviour, production performances – which were investigated according to two main aims.

1) Welfare assessment – to obtain insight into their housing and management welfare conditions of horses reared for meat production and evaluate whether the selected welfare indicators and behavioural activities were influenced by the main causes of concern that regard intensive breeding farms: stocking density and feeding management.

2) Feeding management: gut health, behaviour, production performances – to evaluate the effects of two feeding managements (one based on high amounts of starch vs. one based on high amounts of fibre) on gut health, behaviour and production performances in horses reared for meat production.

3. MATERIALS AND METHODS

3.1. Welfare assessment

The present PhD project was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy, Prot. n. 2202/2019).

Sections 3.2. and 3.3. describe the methods used to answer the first aim of the present PhD project. The methods described in Section 3.2. are adapted from Raspa et al., 2020a [75]. The methods described in Section 3.3. are adapted by Raspa et al., 2020b [71]. Both the studies were carried out in the same horse breeding farm for meat production in Northern Italy. The farm adopts intensive farming methods and sends a total of 2,000 animals to slaughter each year. The horses are housed in group pens situated in a barn with two open sides. Horses have no access to any outdoor paddock area. Pens are enclosed by horizontal metal rail bars, which also delimited the pens at the feed bunk level. One automatic drinker providing tap water is available in each pen. The floor is concrete and covered with barley straw bedding. In the farm there is a total of 24 pens. The number of animals per pen varied, and male and female horses are not separated. Horses are fed twice a day (7:00 a.m. and 6:00 p.m.) with long stem self-produced meadow hay (approximately 6 kg/animal/day) and with an amount of concentrate pelleted feed equal to 8 kg/animal/day – chemical composition (% as fed): crude protein 14.21%, ether extract 3.69%, crude fibre 4.44%, ash 8.30%; starch 49.50%; neutral detergent fibre 17.62%, acid detergent fibre 6.44%, acid detergent lignin 0.73%).

3.2. Welfare assessment: stocking density, feeding management and welfare indicators

3.2.1. Selection of group pens

Considering that the farm contains a total of 24 pens, every second pen was selected for the welfare assessment, providing a total of 12 pens for evaluation by means of seven surveys. At the time of each survey, the horses in each group pen had changed, as had the number of animals it contained. As such, different stocking densities could be evaluated by means of the welfare assessment checklist. Table 2 reports the features of the 12 selected pens, and the median and 25th–75th percentile values regarding the number and the height at the withers of the horses housed within each pen for the seven surveys carried out.

Table 2. Area (m^2) and feed bunk length (m) of the 12 multiple pens evaluated in the 7 surveys. The median values (plus 25^{th}-75^{th} percentiles) for the number and the height (at the withers) of the horses within each pen are reported. Adapted from Raspa et al., 2020a [75].

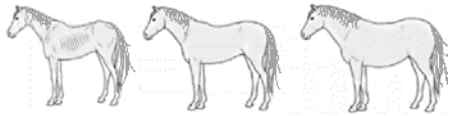
Pen ID	Area of the pen (m^2)	Length of feed bunk (m)	Number of horses median (25^{th}–75^{th})	Height at the withers (cm) median (25^{th}–75^{th})
1	18.1	3.9	2.5 (2–3)	150 (145–150)
2	14.9	3.2	4 (4–4)	140 (137.5–140)
3	20.8	4.6	4 (4–5)	140 (140–140)
4	22.5	4.7	5 (5–6)	140 (140–143.8)
5	16.5	4.0	5 (4–5)	140 (136.3–147.5)
6	27.7	6.7	7 (7–7.75)	140 (130–140)
7	35.0	7.0	9.5 (9–10)	140 (140–150)
8	38.0	7.6	10 (9–11)	130 (130–132.5)
9	36.0	4.8	8 (7.5–8)	147.5 (141.3–153.8)
10	36.8	4.9	10 (9–11)	140 (136.3–140)
11	34.9	4.7	12 (10–13)	140 (140–145)
12	46.5	6.2	15 (14–15)	125 (125–125)

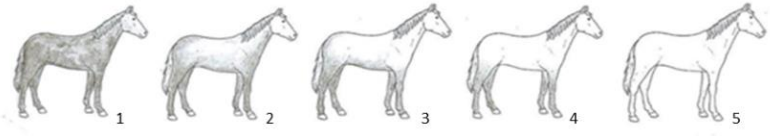
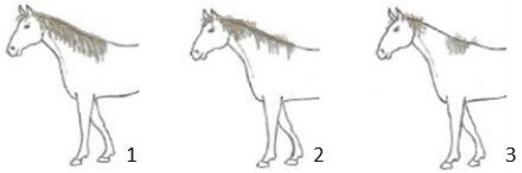
3.2.2. Welfare assessment checklist

A checklist adapted from the AWIN protocol for horses [16] was developed (Table 3) and employed by five equine veterinarians. Prior to the study, the evaluators were trained on the welfare checklist and at the end of the training period, inter-observer reliability was evaluated. The welfare assessment checklist was independently filled by each evaluator during the seven surveys that were carried out from 9:00 a.m. until 12:30 p.m.

Table 3 shows the welfare assessment checklist developed and used by the evaluators. The checklist contained four sections, each regarding one of the four welfare principles of the Welfare Quality® approach: good feeding, good housing, good health, and appropriate behaviour. The welfare indicators included resource-based, management-based, or animal-based indicators as described in the following paragraphs (3.2.2.1., 3.2.2.2., 3.2.2.3., 3.2.2.4.).

Table 3. Welfare assessment checklist used in each of the seven surveys. The checklist is divided into four sections corresponding to the Welfare Quality® principles: good feeding, good housing, good health, and appropriate behaviour. Each principle is measured using specific resource-based, management-based and animal-based indicators. Each section is accompanied by detailed guidance notes and photographs illustrating the scores. Adapted from Raspa et al., 2020a [75].

Welfare principles	Welfare criteria	Welfare indicators	Score	Notes
Good feeding	Appropriate nutrition	N of horses within the group pen	□	
		BCS[1]	□ N of horses scored as Thin … □ N of horses scored as Normal … □ N of horses scored as Fat …	 Thin Normal Fat
		Length of the feed bunk	□ ..... m	Consider as adequate a space at the feed bunk of at least 1 m per horse (m/horse)
		Space allowance per horse at the feed bunk (m/horse)[2]	□ Adequate □ Inadequate	
	Absence of prolonged thirst	Water availability[3]	□ Adequate □ Inadequate	Consider the functioning of the automatic drinkers
		Water point cleanliness[3]	□ Clean: Bowl and water are clean □ Partly dirty: Bowl is dirty but water is clean □ Dirty: Bowl and water are dirty	 Clean Partly dirty Dirty
Good housing	Comfort around resting	Bedding quantity[4]	□ Adequate □ Inadequate	 Adequate (100% of covered floor) Adequate (≥70% of not covered floor) Inadequate (>30% of not covered floor

<table>
<tr><td rowspan="7"></td><td rowspan="2"></td><td>Bedding cleanliness[5]</td><td>□ Adequate
□ Inadequate</td><td colspan="2"> 
Adequate
(≥70% of clean bedding)</td><td colspan="2"> 
Inadequate (>30% of dirty bedding)</td></tr>
<tr><td>Coat cleanliness[6]</td><td>□ N of horses scoring 1 …
□ N of horses scoring 2 …
□ N of horses scoring 3 …
□ N of horses scoring 4 …
□ N of horses scoring 5 …</td><td colspan="4">  </td></tr>
<tr><td rowspan="2">Thermal comfort</td><td>Environmental temperature (°C)[7]</td><td>□ Adequate
□ Inadequate</td><td colspan="4">Environmental temperature is considered adequate if it ranges between +5-+25°C</td></tr>
<tr><td>Environmental humidity (%)[7]</td><td>□ Adequate
□ Inadequate</td><td colspan="4">Environmental humidity is considered adequate if it ranges between 60-80%</td></tr>
<tr><td rowspan="3">Ease of movement</td><td>Area of the pen (m^2)</td><td>□ ..... m^2</td><td rowspan="3" colspan="4"><table><tr><td>Medium height at the withers</td><td>< 120 cm</td><td>120-148 cm</td><td>148-162 cm</td><td>162-175 cm</td></tr><tr><td>Available space per horse (m^2/horse)</td><td>5.5 m^2</td><td>7 m^2</td><td>8 m^2</td><td>9 m^2</td></tr></table></td></tr>
<tr><td>Medium height at the withers of the horses within the pen</td><td>□ …… cm</td></tr>
<tr><td>Stocking density (m^2/horse)[8]</td><td>□ Adequate
□ Inadequate</td></tr>
<tr><td rowspan="2">Good health</td><td rowspan="2">Absence of injuries</td><td>Integument alterations[3]</td><td>N of horses within the pen that present integument alterations …….</td><td colspan="4">Consider integument alteration: area of alopecia, skin lesions as superficial would or deep wound, tumefaction, and swelling</td></tr>
<tr><td>Mane condition[9]</td><td>□ N of horses with a mane score of 1
…
□ N of horses with a mane score of 2
…
□ N of horses with a mane score of 3
…</td><td colspan="4">  </td></tr>
</table>

		Tail condition[9]	□ N of horses with a tail score of 1 … □ N of horses with a tail score of 2 … □ N of horses with a tail score of 3 …	
		Swollen joints/signs of lameness[10]	N of horses within the pen that present swollen joints/signs of lameness ……	Focus attention on distal legs, the shape of the hoof and the animals' movements
	Absence of diseases	Coughing[10]	N of horses within the pen with coughing……	Evaluate coughing together with breathing assessment
		Abnormal breathing[10]	N of horses within the pen with abnormal breathing……	Consider breathing abnormal if the horse shows any of the following signs: flared nostrils, extended head and neck, increased respiratory rate, or asynchrony between movements of the chest and the abdomen
		Discharges[10]	N of horses within the pen with discharges……	Consider nasal and ocular discharges
		Consistency of faeces[11]	□ Normal □ Abnormal	Normal   Abnormal
	Absence of pain and pain induced by management procedures	State of the awareness	N of horses within the pen with an abnormal state of the awareness ……	State of awareness is considered abnormal if horses appear: apathetic, depressed, alarmed, in a state of stupor
Appropriate behaviour	Expression of social behaviour	Mutual grooming	N of horses within the pen ……	Body cleaning is performed by one horse towards a conspecific or reciprocally
		Playing	N of horses within the pen ……	Horse plays alone or with other horses. It includes: playing with structural parts of the pen, locomotor play; play fighting
	Expression of other behaviours	Feeding	N of horses within the pen ……	Horse eats hay, straw or feedstuff in the trough or on the ground
		Watching	N of horses within the pen ……	Horse is in a standing position. The expression is attentive, observing the surroundings
		Resting in standing position	N of horses within the pen ……	Horse is in a standing position. The expression is relaxed
		Resting in lying position	N of horses within the pen ……	Horse is lying on the ground in sternal position with the limbs flexed below the body or in lateral position with extended limbs
		Sexual behaviours	N of horses within the pen ……	Stallion sniffs or bites the mare's genitals. The stallion mounts the mare

	Aggressive behaviours	N of horses within the pen ……	They include: snaking (horse stretches its neck towards a conspecific with ears pinned back, threatening to bite); kicking (horse makes a kicking movement towards another horse with one or both hind limbs); biting (horse touches the body of another horse using its teeth whilst its ears are turned backwards).
	Stereotypic behaviours	N of horses within the pen ……	Horse presents stereotypic behaviour: oral and/or locomotor stereotypic behaviours

[1] BCS was scored as thin, normal, or fat on the basis of the visual appraisal of the shape of each animal. [2] Space allowance at the feed bunk was considered adequate if it allowed at least 1 m per horse, as per the suggestions provided by the Code of Practice for the Care and Handling of Equines [51]. [3] Scores adapted from Animal Welfare Indicators (AWIN) welfare assessment protocol for horses [16]. Water availability was assessed adequate when automatic drinkers were functioning. [4] A specific scoring system was developed by the authors to evaluate bedding quantity. Bedding quantity was scored as adequate if ≥70% of the floor was covered by bedding. Bedding quantity was scored as inadequate if >30% of the floor was not covered by bedding. [5] A specific scoring system was developed by the authors to evaluate bedding cleanliness. Bedding cleanliness was scored as adequate if ≥70% of the bedding was clean, and inadequate when >30% of the bedding was dirty. [6] Specific 5-point scoring system developed for the assessment of coat cleanliness: 1: coat completely dirty; 2: dirty limbs, abdomen, barrel, flanks, and neck; 3: dirty limbs, and abdomen; 4: dirty limbs; 5: coat completely clean. [7] Scores adapted from Wageningen UR Livestock Research Welfare Monitoring System Assessment protocol for horses [86]. Temperature was considered adequate when it was within the horse's thermoneutral zone (+5 to +25 °C). Relative humidity was deemed to be adequate when the values ranged from 60 to 80%. [8] Stocking density was considered adequate according to the indications reported in the associated guidance notes adapted from the AWIN protocol in the section for group-housed horses [16] (i.e., if horses are assessed to measure between 120 and 148 cm at the withers, a minimal space of 7 m^2/horse is required to be considered adequate). [9] Specific 3-point scoring system defining mane and tail condition: 1: mane/tail are in good condition for their entire length; 2: areas of broken and/or absent mane or tail hair, but intact skin; 3: areas of broken and/or absent mane or tail hair and damaged skin. [10] Scores adapted from AWIN welfare assessment protocol for horses [16]. [11] Faeces were scored as normal if the shape of the faeces was conserved.

3.2.2.1. Good feeding

The welfare principle "good feeding" was described by its two welfare criteria: "appropriate nutrition" and "absence of prolonged thirst".

To assess "appropriate nutrition", the body condition score (BCS) was rated and recorded. The BCS is the only welfare indicator used in the AWIN protocol to describe the welfare criteria "appropriate nutrition". It is scored using a 5-point scale [87] in which the nutritional status of an animal is assessed through observation and palpation of anatomical key areas. In the present study, the BCS of the horses was scored as "thin", "normal" or "fat" by means of the visual appraisal of the animals' shape alone, since it was not possible to touch the horses during the assessment (see Table 3, with associated guidance notes and illustrative photographs). The number of horses per pen judged as "thin" was recorded and used in the statistical analysis. This study also considered space allowance at the feed bunk as a welfare indicator of "appropriate nutrition" since easy access to feed troughs must be guaranteed to ensure the welfare of animals in production systems [88]. Space allowance at the feed bunk (m/horse) was calculated by dividing the length of the feed bunk (meters) by the number of horses within the pen.

The welfare criterion "absence of prolonged thirst" was assessed by considering water availability and water point cleanliness. Water availability was assessed by evaluating the correct functioning of the automatic drinkers. Water point cleanliness was scored as suggested by the AWIN protocol; specifically, the drinkers were scored "dirty" if both the bowl and water were dirty (i.e., the presence of organic materials, such as feed, soil or faeces); "partly dirty" if the bowl was dirty but the water clean, or "clean" if both bowl and water were clean (see Table 3 with associated guidance notes and illustrative photographs). The frequency (%) of the automatic drinkers scored as adequate or inadequate was calculated and used in the statistical analysis.

3.2.2.2. Good housing

The welfare principle "good housing" includes the welfare criteria "comfort around resting", "thermal comfort" and "ease of movement".

Comfort around resting was evaluated by considering the two welfare resource-based indicators, "bedding quantity" and "bedding cleanliness", as used in the AWIN protocol, plus "coat cleanliness".

The AWIN protocol scores the former two indicators in a qualitative manner only through the use of pictures. Here, in order to achieve a more standardised method, we developed a specific scoring system to evaluate bedding quantity and cleanliness. Bedding quantity was scored as adequate when ≥70% of the floor was covered (defined in the AWIN protocol as "sufficient bedding material"), and inadequate if >30% of the floor was not covered (defined in the AWIN protocol as "no bedding material" and "insufficient bedding material"; see Table 3 with its detailed guidance notes and photographs illustrating

the scores). Bedding cleanliness was scored as adequate if ≥70% of the bedding was clean (defined in the AWIN protocol as "clean bedding material") and inadequate when >30% of the bedding was dirty (defined in the AWIN protocol as "dirty bedding material"; see Table 3 with its detailed guidance notes and photographs illustrating the scores). For the statistical analysis, bedding quantity and bedding cleanliness were expressed as frequencies (%) of scores.

Coat cleanliness was also taken into consideration for the assessment of "comfort around resting". We decided to evaluate this welfare indicator as it reflects the environmental conditions in which the animals are kept. A specific 5-point scoring system was designed to assess coat cleanliness (see Table 3 with its detailed guidance notes and photographs illustrating the scores). Horses were assigned a score of 1 if they were completely dirty; a score of 2 if they presented dirty limbs, abdomen, barrel, flanks and neck; a score of 3 for dirty limbs, and abdomen; a score of 4 for dirty limbs only; a score of 5 for a completely clean horse. A coat cleanliness score of 1, 2 or 3 was rated "dirty". The number of horses per pen rated as dirty was used for the subsequent statistical analysis.

For the welfare criterion "thermal comfort", since it was not possible to evaluate this parameter by examining whether the animals that showed clinical signs of thermal stress, as suggested in the AWIN protocol, thermal comfort was instead evaluated through the measurement of environment temperature (°C) and relative humidity (%). These measurements were taken in front of each pen using a digital thermometer and hygrometer. According to the Wageningen UR Livestock Research Welfare Monitoring System [86], the temperature was considered adequate when it was within the horse's thermoneutral zone (+5 to +25 °C); and relative humidity was deemed to be adequate when the values ranged from 60% to 80%.

The welfare criterion "ease of movement" should regard the quality of the exercise horses are able to partake in. The AWIN protocol describes this management-based indicator by referring to the possibility for animals to spend part of their day performing activities in outdoor areas. Since it was not possible to apply this welfare indicator in the evaluation of animals kept in a production system, we decided to evaluate each pen's area (m^2) and stocking density (m^2/horse) to gain some data pertaining to the animals' possibility for "ease of movement". Once the area of a pen was calculated, it was then divided by the median height of the horses, measured to the withers, within the pen. As we were not able to touch the animals, a laser meter was used to measure the height of animals at the withers. Measurements were conducted for the tallest and the shortest horse in order to ascertain the height range for the horses within a pen. The measurement was made at the moment in which the animal was standing in a position that was parallel to the wall or to the horizontal metal rail bars. The stocking density was considered adequate or inadequate according to the indications provided in the AWIN protocol in the section adapted for group-housed horses [16]. Accordingly, if animals were assessed to measure between 120 and 148 cm at the withers, a minimal space of 7 m^2/horse was required, whereas

if the heights ranged between 148 and 162 cm, an adequate space allowance should not be less than 8 m^2/horse.

3.2.2.3. Good health

The welfare principle "good health" includes three welfare criteria: "absence of injuries", "absence of diseases", and "absence of pain and pain induced by management procedures".

"Absence of injuries" is described by evaluating the animal-based indicators "presence of integument alterations" and "presence of swollen joints—signs of lameness", as well as "mane condition" and "tail condition".

The presence of integument alterations was evaluated by recording the extent of visible areas of alopecia, skin lesions (as superficial or deep wounds), tumefaction, and swelling. Since it was not possible to approach the animals, a visual inspection of the body of each animal was performed. In the checklist, the number of horses within each pen presenting at least one visible integument alteration was recorded and used for statistical analysis.

The number of horses presenting visibly swollen joints and/or signs of lameness was recorded. In addition, a visual inspection of the body of each horse within the pen was performed, focusing attention on the distal limbs, the shape of the hooves, and the animals' movements.

In our assessment of the welfare criterion "absence of injuries", we decided to introduce two additional animal-based indicators on the basis of their initial observations of the animals; they were mane condition and tail condition. We decided to include these welfare indicators as they seemed to reflect the specific housing and management features of this kind of farm. In particular, the observation of alterations to the mane and/or tail seemed to constitute a specific "occupational ailment" in this specific context. A specific 3-point scoring system was defined for both mane and tail condition: a score of 1 indicated good mane/tail condition for their entire length; a score of 2 indicated areas of broken and/or absent mane/tail hair, but with the skin intact; and a score of 3 indicated a damaged mane/tail with areas of broken and/or absent mane or tail hair and injured skin (see Table 3 with its detailed guidance notes and the photographs illustrating the scores).

The welfare criterion "absence of diseases" was assessed using four animal-based welfare indicators: "coughing", "abnormal breathing", "discharges", and "consistency of faeces". Coughing and abnormal breathing were recorded as the number of horses presenting these symptoms. To evaluate breathing, the head and the flanks of each horse were observed. Breathing was considered abnormal when at least one of the following clinical signs were observed: flaring of the nostrils, extended head and neck, increased respiratory rate, or asynchrony between movements of the chest and the abdomen. The number of horses within the group pen coughing or with abnormal breathing was recorded and used in the statistical analysis. Nasal and ocular discharges were evaluated by observation. This assessment

was performed at the same time as the assessment for coughing and abnormal breathing. Once again, the number of horses within the group pen presenting these clinical signs was recorded.

The consistency of faeces was considered by evaluating the shape of the faeces present in the bedding of each group pen and recorded as normal and/or abnormal. Faeces were scored as abnormal if the shape of the faeces was not conserved. For statistical analysis, the frequency (%) of group pens containing abnormal faeces was calculated.

To assess the welfare criterion "absence of pain and pain induced by management procedures", the indicator "state of awareness" was evaluated. The AWIN protocol recommends the use of the Horse Grimace Scale that assesses equine facial expressions for the assessment of pain; however, this was not deemed feasible in the present study, thus the concept of state of awareness was introduced as an alternative. This involved observing the animals and noting whether they presented any symptoms of an "abnormal" state of awareness, which includes the adoption of a depressed or an alarmed stance, paying no attention to the surrounding environment and an inadequate response to stimuli, such as light, noise and the presence of people. The number of horses per pen that presented an abnormal state of awareness was recorded and used in the statistical analysis.

3.2.2.4. Appropriate Behaviour

To assess the welfare principle "appropriate behaviour", the following welfare indicators were considered (as measures of the welfare criteria "expressions of social behaviour" and "expressions of other behaviours"): feeding, watching, mutual grooming, resting in a standing position, resting in a lying position, playing, sexual behaviours, aggressive behaviours, and stereotypic behaviours (licking, crib-biting, weaving, head nodding, wood chewing; see Table 3 with its detailed guidance notes). To assess these indicators, all five evaluators simultaneously observed the horses within a single pen. They were positioned at different positions outside the pen at a maximum distance of 5 m from the horses. The welfare assessment started 5 min after the placement of the evaluators, who remained still and quiet to allow the horses to become accustomed to their presence. A methodology was adapted that involved observing the horse situated the furthest to the left in the pen, then moving to the animal situated to its right, and so on. The number of horses displaying each specific behaviour was recorded and used for statistical analysis.

3.2.3 Statistical analysis

The data pertaining to the individual group pens were assigned to one of two groups on the basis of their stocking density (m^2/horse). The median stocking density was calculated in order to divide the data into two groups, depending on whether they were housed at a low stocking density (LSD^{50th}; i.e., at or above the 50th percentile) or a high stocking density (HSD^{50th}; below the 50th percentile). The 75th percentile value was also calculated, and the animals again divided into low or high stocking

density groups depending on whether they were housed at or above, or below the 75th percentile stocking density (LSD^{75th} and HSD^{75th}, respectively).

Data were analysed using IBM SPSS® Statistics 21.0 software (SPSS Inc., Chicago, IL, USA) to identify any differences between the groups divided according to the stocking density cut-off values. The Shapiro–Wilk test was used to assess whether the data were distributed according to a normal distribution. Since the data were not normally distributed, the Mann–Whitney U and the Fisher's exact tests were applied. A p-value < 0.05 was considered significant to infer that differences between the groups were related to the stocking density.

The inter-observer reliability of the expert evaluators in their assessment of welfare indicators was evaluated by means of the Cohen's kappa coefficient (K).

Dichotomous variables (bedding cleanliness, bedding quantity, consistency of faeces, water point cleanliness) were expressed as frequencies (% of group pens). The other welfare indicators (i.e., the nondichotomous variables) were expressed as the number (N) of horses within each group pen presenting a specific score or health condition or performing a specific behaviour.

3.3. Welfare assessment: stocking density and behavioural activities

3.3.1. Selection of group pens

The inclusion criteria for pen selection were based on the stocking densities. Moreover, to be included in the study, horses within group pens needed to be homogenous for breed, age, height at the withers, and time since arriving at the farm. This latter criterion ensured that all the horses were equally accustomed to the housing and management conditions of the breeding farm.

Only three group pens in the barn met these criteria. Table 4 reports the number of horses, pen area (m^2), stocking density (m^2/horse), and feeding space per horse at the feed bunk (m/horse) for each pen. A total amount of 22 horses (19 males and 3 females) with a height at the withers ranging between 140 and 150 cm were involved in the study. All the horses belonged to the Comtois breed, and their mean age (±standard deviation) was 22 ± 2 months. All the animals had spent six weeks in the barn before being involved in the present study.

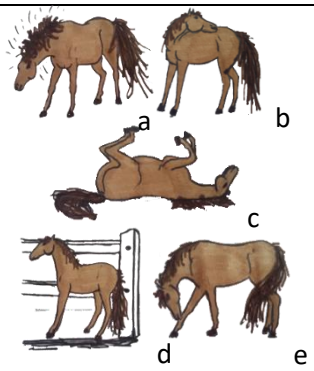
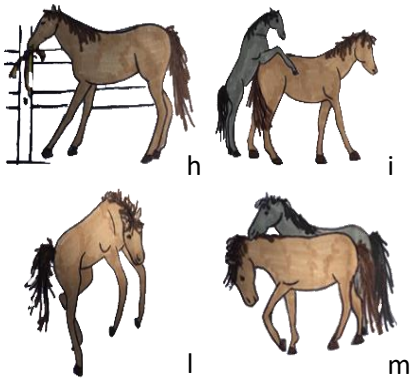
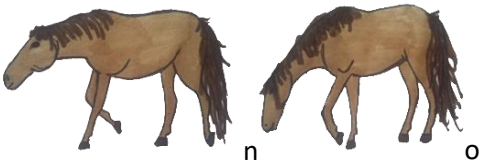
Table 4. The number (N) of horses, pen area (m^2), stocking density (m^2/horse), and space at the feed bunk (m/horse) within each pen are reported. Adapted from Raspa et al., 2020b [71].

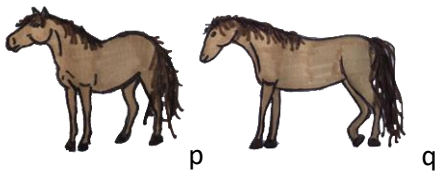
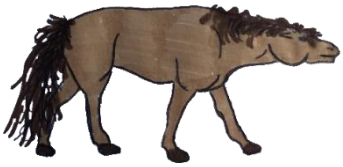
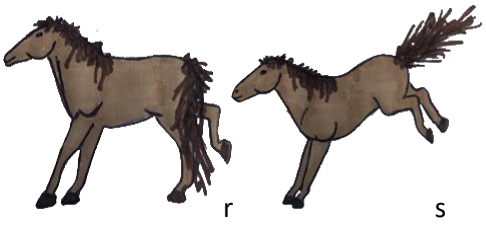
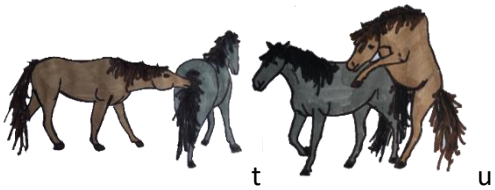
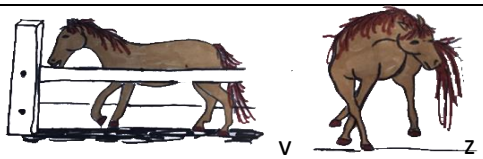
Id Pen	N of Horses	Pen Area (m^2)	Stocking Density (m^2/horse)	Space at the Feed Bunk (m/horse)
A	8	35.00	4	0.88
B	8	36.75	5	0.61
C	6	36.00	6	0.80

3.3.2. Behavioural observations

One 2D camera equipped with infrared light (Hikvision IP 3.0 Megapixel—NDV Network Video Recorder Hikvision 7600 Series) was installed on each selected pen. The cameras were oriented so that the horses were never out of sight. Observations were recorded for 72 h, corresponding to three consecutive days (24th to 26th November). Videos were evaluated by two trained observers using an ethogram that was specifically developed (Table 5). Before the behavioural data were collected, the observers underwent specific training to be ensure an adequate degree of concordance. Accordingly, inter- and intra-observer reliability were evaluated. The ethogram consisted of 13 mutually exclusive behavioural activities, meaning that the horse could only be doing one of the named activities at any one time (as suggested by McFarland and Sibly, 1975 [89]). The observations of behavioural activities were performed using scan sampling [90,91]. The behaviours expressed by each horse in the pens were assessed by scan sampling at 15 min intervals throughout the 72 h observation period.

Table 5. Description and illustrations of the selected mutually exclusive behaviour activities. Adapted from Raspa et al., 2020b [71].

Activities	Descriptions	Illustrations
Self-grooming	The horse performs body cleaning by himself. It includes: shaking the entire body or a part of it (a); nibbling or licking the coat hair (b); rolling on the ground (c); rubbing parts of the body against objects (d) or other parts of the body (e.g., rubbing the muzzle against the limbs) (e).	
Mutual grooming	Body cleaning is performed reciprocally or by one horse towards a conspecific.	
Lying	The horse is lying on the ground in the sternal position with the limbs flexed below the body (f) or in lateral position with extended limbs (g).	
Playing	The horse plays alone or with other horses. It includes: play with structural parts of the pen (h), sexual play (i), locomotor play (l), and play fighting (m).	
Locomotion	The horse moves inside the pen by taking steps; the neck is in a horizontal position (n) or lowered to the ground to sniff (o).	
Feeding	The horse eats hay, straw or feedstuff in the trough or on the ground.	
Drinking	The horse drinks.	

Standing	The horse is in quadrupedal station. The expression is relaxed or attentive. It includes: "standing alert" (p) and "standing relaxed" (q).	 p q
Snaking	The horse stretches its neck towards a conspecific with the ears turned backwards, the lips are often closed and the body is in a dominant position.	
Kicking	The horse lifts one (r) or both hind limbs (s) off the ground and quickly stretches it/them towards a conspecific, aiming to hit him.	 r s
Biting	The horse quickly opens and closes its mouth and its teeth touch the body of a conspecific, aiming to bite him. The ears are turned backwards.	
Sexual behaviour	The stallion sniffs or bites the mare's genitals (t). The stallion mounts the mare: erection and penetration are present (u).	 t u
Stereotypic behaviour	The horse expresses a stereotyped behaviour: both oral (v) and locomotor stereotypes (z) are considered.	 v z

3.3.3. Statistical analysis

Statistical analyses were performed using JMP v14.3 (SAS Institute Inc., Cary, NC, USA). The inter- and intra-observer reliability of the trained observers was evaluated by means of the Cohen's Kappa Coefficient (K).

Each pen was considered as a statistical unit. In order to investigate the time-budget pattern, we used the frequency (%) ± SD for the selected behavioural activities. Frequencies were calculated for each day of observation, and data were collected for:

- 24 h periods (%/24 h);
- 12 daylight hours (8:00 am–8:00 pm) (%/daylight hours);
- 12 night hours (8:00 pm–8:00 am) (%/night hours).

3.3.3.1. Correlations between time-budget and stocking densities

Bivariate analysis was used to investigate the effect of stocking density (categorical predictors, 4, 5 and 6 m^2/horse) on the behavioural activity frequencies (%/24 h; %/daylight hours; %/night hours). Relationships were analysed using the Pearson's correlation coefficient (r, 1 or −1 depending on whether the variables are positively or negatively related [92]). The r coefficient values for correlation were interpreted according to Prior and Haerling, 2014 [93]: very strong correlation (±0.91 to ±1.00); strong correlation (±0.68 to ±0.90); moderate correlation (±0.36 to ±0.67); weak correlation (±0.21 to ±0.35); and negligible correlation (0 to ±0.20). The probability of correlation (p-value) was calculated and Pearson correlations were considered significant at $p \leq 0.05$.

3.3.3.2. Overall time-budget and time frame

We calculated the mean frequency value for each behavioural activity for the 72 h observation period (overall time-budget) considering all 22 horses. The overall time-budget of each behavioural activity engaged in by the horses was further divided according to 6 time intervals (00:00–04:00; 04:00–08:00; 08:00–12:00; 12:00–16:00; 16:00–20:00; 20:00–24:00) as described by Boyd et al., 1988 [94]. In particular, data for the time-budget of the main expressed behavioural activities (feeding, lying, standing, and locomotion) performed by young Przewalski horses (age range: 2 to 3 years) were adapted from Boyd et al. [94] in order to compare the behavioural activities between horses reared for meat production and wild-living horses.

3.4. Feeding management: gut health, behaviour, production performances

The following sections describe the methods used to answer the second aim of the present PhD project. The feeding trial was carried out in the same farm described in Raspa et al., 2020a [75] and Raspa et al., 2020b [71]. The experimental protocol was designed according to the guidelines of the current European Directive (2010/63/EU) on the care and protection of animals and, as stated before, approved by the Ethical Committee of the University of Turin (Italy) (Prot. N. 2202/2019).

3.4.1. Animals, management and diet

The feeding trial involved nineteen horses of the Bardigiano breed (12 females and 7 males) aged 14.3 ± 0.7 months (mean ± standard deviation, SD). According to Raspa et al., 2021 [95], upon their arrival at the farm, horses were treated against internal parasites (1.29 g/100 kg BW; Equalan duo; Merial Animal Health, Harlow, UK). During the subsequent two weeks, they were kept together in an outdoor paddock and fed the same grass hay which was provided ad libitum. After the adaptation period, horses were randomly divided into two group pens (7m x 9m), which assured a space allowance of at least 6 m^2 per animal. The group pens were located side by side (Figure 6), each of which was enclosed by horizontal metal rail bars, delimiting the pens at the feed bunk level.

Figure 6. The two group pens involved in the feeding trial. On the left, the group pen fed the high starch (HS) diet; on the right the group pen fed the high fibre (HF) diet.

The horses received the same hay but a different concentrate feed (described in Table 6). One group of horses was individually fed with a high starch and sugar cereal grain-based complementary feed and received a high starch diet (HS; 43% hay plus 57% cereal grain-based pelleted feed); the other group was individually fed with a fibre-rich complementary feed and received a high fibre diet (HF; 70% hay plus 30% pelleted fibrous feed). The complementary feeds were supplied twice a day (7.00 am and 6.00 pm) and hay was provided estimating the hay consumption to be fed 6 kg/animal/ day for the HS group and 8 kg/animal/day for the HF group. The complementary feeds were gradually increased to reach the final amount during the last 72 days of the fattening period; more details about the feeding trial are reported in Raspa et al., 2021 [95]. The proximate analysis of the hay, the different complementary feeds used and the daily nutritional composition of the diets (HS and HF) are reported in Table 6 and Table 7, respectively.

Table 6. Chemical composition (% as fed) of hay and pelleted feed. Adapted from Raspa et al., 2021 [95].

	Hay	High starch feed HS [1]	High fibre feed HF [2]
DM [3]	89.81	89.91	90.59
Crude protein	6.62	14.21	19.77
Ether extract	1.03	3.69	5.06
Crude fibre	30.04	4.44	11.53
Ash	6.23	8.30	10.78
Starch	0.27	49.50	19.11
NDF [4]	55.20	17.62	27.10
ADF [5]	35.06	6.44	15.28
ADL [6]	4.01	0.73	1.98

[1] High starch; [2] High fibre; [3] Dry matter; [4] Neutral detergent fibre; [5] Acid detergent fibre
[6] Acid detergent lignin

Table 7. Overall nutritional composition of the diets (referred to the total daily diet: hay plus pelleted feed) as fed to the high cereal grains group (HS) and the high fibre group (HF) during the fattening period (72 days). Adapted from Raspa et al., 2021 [95].

Nutritional components	HS [1]	HF [2]
Kg hay/animal/day	6	8
Kg pelleted feed/animal/day	8	3.5
Forage intake/kg BW (%)	1.73	2.32
DM intake (kg)	12.60	10.25
Net energy (MJ) [3]	95.88	53.58
Crude protein (g)	1557.20	1159.60
Digestible Crude Protein (g MADC)	1177.66	723.25
Crude fat (g)	285.40	192.70
Fat contribution to total energy content provided (%)	8.39	10.14
Ash (g)	901.8	904.4
Calcium (g)	377.80	108.22
Phosphorous (g)	188.60	35.79
Lysine (g)	48	76.50
Vitamin E (mg)	399.68	1105
Selenium (mg)	0.48	1.72

[1] High starch; [2] High fibre; [3] Net energy was calculated according to Martin-rosset, 2015

Animals were weighed at the beginning and at the end of the trial in order to calculate the average daily gain in bodyweight. All horses were weighed at the same time of the day when they arrived on the farm and the evening before slaughter after the evening meal. At the end of the fattening period, all animals were slaughtered. The commercial authorized abattoir was 7 km from the horse farm and took less than 25 minutes travelling time to reach. All the procedures carried out during this phase were supervised by the official veterinarian and conducted according to the European Union regulations (EU Regulation 2009/853 and EU Regulation 627/2019). In the sections below are described the sampling procedures adopted to compare the effects of the conventional feeding management (HS) with the experimental feeding management (HF) on gut health (Section 3.5.); behaviour (Section 3.6.) and production performances (Section 3.7.)

3.5. Feeding management: gut health

3.5.1. Sample collection

Immediately after horses were slaughtered, stomachs were isolated from the intestine and scored according to the methods described in the section 3.4.2.

At the same time, samples of the gut content were taken from different intestinal compartments. The intestinal compartments were the following (Figure 7):

a) the small intestine (SI) – a pooled chyme sample from duodenum, jejunum and ileum was collected as a consequence of the fact that there was too little material available from those individual intestinal compartments.

b) the apex of the caecum (CAE),

c) the sternal flexure (SF),

d) the pelvic flexure (PF),

e) the right dorsal colon (RDC)

f) the rectum (RE).

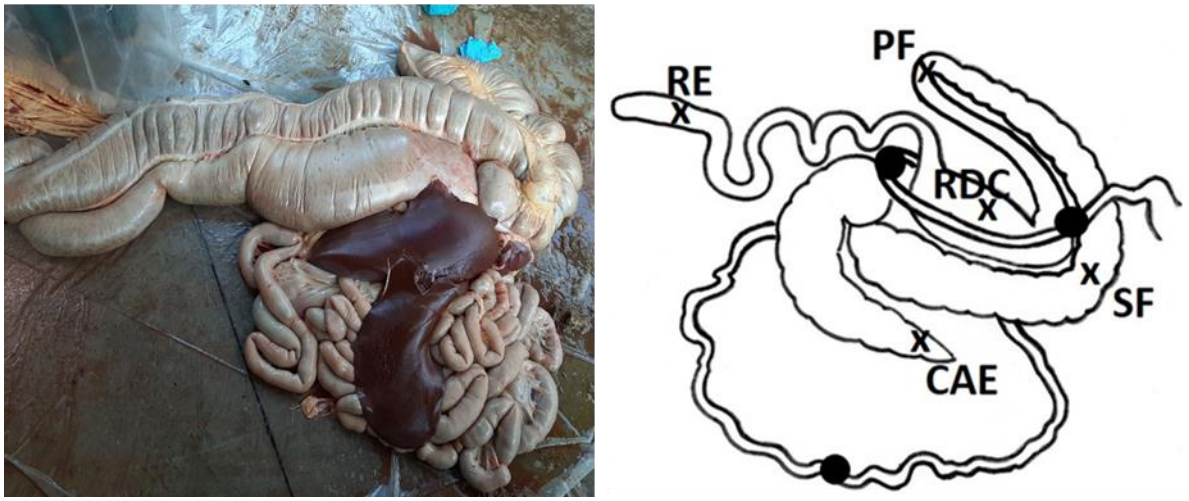
Each intestinal compartments were identified and clamped with ligatures before they were opened for sampling. Samples were differently collected and stored according to the laboratory analyses which were performed after sample collection, as described in the sections 3.5.3., 3.5.4., 3.5.5.

Moreover, intestinal segment samples (approximately 5 cm in length) of duodenum (DU, jejunum (JEJ), ileum (ILE), CAE, SF, PF, RDC and RE were excised and flushed with 0.9% of saline solution in order to remove all the contents. All the samples were fixed in 10% buffered formalin to proceed after with the morphometric and histopathological analyses, as described in section 3.5.6.

Besides, immediately after evisceration, liver tissue and mesenteric lymph nodes were aseptically collected from the packed viscera by a trained operator and placed into sterile bags. Samples were transported to the laboratory at 4°C for microbiological analysis and processed within one hour, as

described in section 3.5.7. Moreover, 100 g liver sample was frozen at -20°C for subsequent analysis of antioxidant enzymes and oxidation end-products as described in section 3.6.2.

Figure 7. Illustration of the sampling sites. The points indicated the sites of the small intestines that were collected to obtain the pooled sample of duodenum, jejunum and ileum. The crosses indicate the sites in which samples were collected from the other intestinal compartments: CAE=apex of the caecum, SF=sternal flexure, PF=pelvic flexure; RDC=right dorsal colon; RE=rectum.



3.5.2. Scoring of the gastric mucosa lesions

After stomachs were isolated, they were opened by cutting along the great curvature, emptied and gently washed with cold tap water in order to remove all the content. Squamous and glandular regions were evaluated separately given a score from 0 to 4. In particular, the squamous mucosa was scored according to European College of Equine Internal Medicine Consensus Statement [30]. Whereas, the glandular gastric mucosa was scored according to the scoring system proposed by Vondran et al. [96].

3.5.3. Dry matter, organic matter and ash content analyses

The samples from the each selected intestinal compartments (300 ±50 g fresh matter) were collected in pre-identified plastic boxes that were sealed and frozen at –20 °C until analyses. Samples were thawed and dried in a forced-draft oven at 100 °C for 1 h. After temperature was set at 60 °C until to constant weight. Subsequently, they were ground to pass 1 mm sieve in order to determine the dry matter, the organic matter and the ash content according to VDLUFA [97]. Organic matter (OM) was calculated for each material with the following formula: OM=100-Ash-Moisture.

3.5.4. Analysis of particle size

Samples from the each selected intestinal compartments (50 g) – SI excluded since compartment did not contain enough material – were collected in Falcon collection tubes (Falcon Conical Centrifuge Tube, Tewsbury, MA) that were sealed and frozen at –20 °C until analyses.

Particle sizes was determined by wet sieving according to the method described by Vondran et al.,2016 [26]. Briefly, samples were thawed and soaked in beakers with 1 L water overnight prior to sieving. Samples were sieved for 5 minutes with sieves with respective mesh sizes of 8, 4, 2 and 1 mm. Subsequently, the materials remained on each sieve was dried 60 °C for 12 hours and cooled before weighing. The dry amount on each sieve was expressed as a percentage of dry weight of the total sample. The latter was calculated from the weight of the faeces measured before and after drying. The fraction that was washed through the finest sieve (<1 mm) was calculated from the total sample weight minus the sum of the four sieves fraction.

3.5.5. Analysis of volatile fatty acids (VFAs)

After slaughtering, samples for VFAs quantification – one pooled sample of duodenum, jejunum and ileum for the small intestine, one sample from the other selected intestinal compartments of the hindgut - were collected in Falcon collection tubes (Falcon Conical Centrifuge Tube, Tewsbury, MA). Samples were immediately frozen at –20 °C until analysis, that was carried out according to the method described by Guantario et al.,2020 [98]. Briefly, samples from the small intestine (15 g) and from the other intestinal compartments (30 g) were suspended in 50 and 100 ml of 0.1 N H_2SO_4 solution, respectively, homogenized in a stomacher (Lab-Blender 400, Seward, Worthing, UK) for 5 minutes and centrifuged twice at 15,000X g for 10 min at 4 °C. The resulting extracts were filtered through a paper filter and then through 0.22 µm pore siringe filter.

Analyses were performed by HPLC (High Performance Liquid Chromatography) using a Dionex Ultimate 3000 (Thermo Fisher) with autosampler equipped with a 300 × 7.8 mm Aminex HPX-87H (Bio-rad) and a guard-column. Injected samples (30 µL) were isocratically separated in 0.005 N H_2SO_4, at a flow rate of 0.6 mL/min at 41 °C. VFAs were detected by UV at 210 nm, using an external standard curve (4.95–148.5 mg/100 ml succinic acid; 9–270 mg/100 ml lactic acid; 10.5–314.4 mg/100 ml acetic acid; 9.85–285.5 mg/100 ml propionic acid; 9.4–282.1 mg/100 ml butyric acid; 9.5–285.1 mg/100 ml isobutyric acid; 9.1–273.4 mg/100 ml iso-valeric acid; 9.1–273.2 mg/100 ml valeric acid) in 0.1 N H_2SO_4. Total VFAs were expressed as mg/100 ml. Individual VFAs were expressed also as percentage (%) on the total VFAs.

3.5.6. Morphometric and histopathological analyses

The present analyses were performed by the colleagues of the Sector of Pathology at the Department of Veterinary Science of the University of Turin.

Samples were embedded in paraffin wax blocks, sectioned at a 5-µm thickness, mounted on glass slides and stained with Haematoxylin & Eosin (HE). Morphometric analyses were performed on HE cross-sections of duodenum, jejunum and ileum using a computerised image analysis system (Image®-Pro Plus software, 6.0 version, Media Cybernetics, Maryland, USA) coupled to a Nikon DS-Fi1 digital camera

(Nikon Corporation, Minato, Tokyo, Japan) and a light microscope with a 2.5× objective lens. The evaluated parameters were: villus height (Vh, from the tip of the villus to the crypt), villus width (Vw, across the base of the villus, but not including the brush border), crypt depth (Cd, from the base of the villus to the sub- mucosa) and mucosa thickness (form the tip of the villus to the muscularis mucosa). Villus height-to-crypt depth (Vh/Cd) ratio and the villus absorptive surface area ($2\pi \times Vh \times (Vw/2)$ were also calculated [99]. Morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts for each intestinal segment while mucosa thickness was measured in triplicate. Additionally, all the sampled were submitted to histopathological evaluation using a semi-quantitative score (0: absent; 1: mild and multifocal; 2: moderate and disseminated; 3: severe and diffuse). The inflammation and/or GALT activation in the gut was investigated.

3.5.7. Procedures to assess microbiological contamination of mesenteric lymph nodes and liver samples

According to Raspa et al, 2021 [95]. Immediately after slaughter, 100 g liver sample and 100 g of mesenteric lymph nodes processed to assess their microbiological contamination. Mesenteric lymph nodes were processed as described by Webb et al.,2017 [100] and Mainar-Jaime et al.,2013 [101]. Accordingly, samples of mesenteric lymph nodes were aseptically trimmed to remove excess fat and fascia. The trimmed lymph nodes were submerged into boiling water for 3-5 seconds and then flamed using a Bunsen burner for 3 s. Then, they were sterile cut and weighed to obtain 25 g/animal for the detection of *Salmonella* spp., and 10 g/animal for the detection of *E. Coli*.

Liver samples were surfaced flamed before proceeding with deep subsampling. Liver subsamples were then obtained using a sterile scalpel by cutting deep into the organ's tissue. Samples weighing 25 g/animal and 10 g/animal were used for the detection of *Salmonella* spp. and *E. Coli*, respectively. Subsequently, samples were homogenized according to the analyses described in the subsequent sections – 3.7.7.1, 3.5.7.2 and 3.5.7.3.

3.5.7.1. TMABc and Enterobacteriaceae counts

ISO procedures were used for TMABc and *Enterobacteriaceae* counts (ISO 4833-1:2013 and ISO 21528-2:2017, respectively). Briefly, for the detection of TMAB, tissue samples were diluted in Buffered Peptone Water (BPW; CM 509 B, Oxoid, Rodano, Milan) and appropriately plated onto Plate Count Agar (PCA CM 0325 Oxoid, Rodano, Milan), then incubated at 31°C for 48 hr. For the detection of Enterobacteriaceae, Violet Red Bile Glucose Agar (VRBG agar CM 0485 Oxoid, Rodano, Milan) was streaked and incubated at 37°C for 48 hr. The results are expressed in CFU/g.

3.5.7.2. Isolation of *Salmonella spp.*

The isolation of *Salmonella* spp. was carried out in accordance with ISO 6579-1:2017. After pre-enrichment in BPW for 24 h at 37°C, 1 mL and 0.1 mL of each pre-enrichment solution was inoculated into 10 ml of Selenite Cystine Broth base (CM 0699, Oxoid , Rodano, Milan) and 10 mL of Rappaport-Vassiliadis Broth (CM 669 B, Oxoid , Rodano, Milan), respectively, and then incubated at either 37°C (Selenite Cystine Broth) or 41°C (Rappaport-Vassiliadis Broth) for 24 h and plated onto selective Xylose Lysine Deoxycholate (XLD) Agar (CM 0469, Oxoid, Rodano, Milan) and Hektoen Enteric Agar (HEA) (CM 0419, Oxoid, Rodano, Milan). Following 24 h incubation, suspect colonies of *Salmonella* spp. were tested by inoculation into Kligler iron agar (CM0033, Oxoid, Rodano, Milan).

3.5.7.3. Isolation of *Escherichia Coli*

The isolation of *E. Coli* spp. was performed as described in ISO 16649-1,2:2001 using tryptone bile x-glucuronide (TBX) medium (Oxoid Ltd, Basingstoke, UK). Plates were incubated at 41°C per 24 hr. Suspected colonies of *E. Coli* spp. were then tested using API 20 Enterobacteriaceae (API 20E) strips (BioMérieux Italia, Bagno a Ripoli, Florence).

3.5.8. Statistical analysis

Data were statistically analysed using the software JMPpro v16 (SAS Institute Inc., Cary, NC). Each parameter was tested for normal distribution using the Shapiro-Wilk test and normalized, when necessary, by box-cox transformation [102]. A linear mixed effect model was constructed setting dietary treatment, sex and their interaction as model fixed effects. Then, each horse within sex and diet was considered as experimental unit and used as random variable for all analyses. Least squares means were separated using T-Student's adjusted *p*-values when at least a tendency F-test ($p \leq 0.10$) was detected in the fixed effect interaction term [42,43].

3.6. Feeding management: behaviour

3.6.1. Behavioural observations

During the experimental trial, the two group of horses were video-recorded with one 2D camera equipped with infrared light (D-Link DSH-C310 180°, Full HD). Behavioural observations were recorded for 96 h, thus for four consecutive days (17th to 20th September 2019). The videos were evaluated by one trained operator expert in equine field by using the ethogram published by Raspa et al., 2020b [71]. The observations of behavioural activities were carried out by means of scan sampling [91] at 10 min intervals throughout the 96 h observation period.

3.6.2. Statistical analysis

Statistical analyses were carried out using JMP v15.1 (SAS Institute Inc., Cary, NC, USA). The mean frequency (%) for each behavioural activity considering each 24 h observation period was calculated according to the two groups of horses (HS vs. HF). All the behavioural data were checked for normality, employing the Shapiro–Wilk test. A $p>0.05$ was considered indicative of a normal distribution. Data were reported as mean ± standard error of the mean (± SEM) or median (plus 25^{th}–75^{th} quantiles) depending of normal or not normal distribution, respectively.

Normally distributed values were analysed by one–way ANOVA to determine the differences between the two groups of horses (HS vs. HF). The variables without normal distribution were tested by the Wilcoxon non-parametric test. The significance level was set at $p>0.05$.

3.7. Feeding management: production performances

All the methods described in this present section have been adapted from Raspa et al., 2021 [95]. All the analyses described here were carried out by Professor Pasquale De Palo and his colleagues of the Department of Veterinary Medicine of the University of Bari, Italy.

3.7.1. Analysis of *Longissumus thoracis et lumborum* muscle samples

The *Longissimus thoracis et lumborum* muscle of the right half-carcass was immediately refrigerated at 4°C and sampled at the $17/18^{th}$ thoracic vertebrae level after 24 hours of storing at low temperature. One sample was processed for the analyses of muscle characteristics as described below in subsection 2.7.1.1.; and one aliquot was stored at -20°C until the subsequent analysis of its chemical composition and fatty acid profile as described below in subsections 2.7.1.2. and 2.7.1.3., respectively.

3.7.1.1. Muscle characteristics

Forty-eight hours after slaughtering, the rheological characteristics of muscle samples were assessed. pH measurement was performed using a portable pH meter with a glass electrode shaped to facilitate meat penetration (Carlo Erba pH 710; Carlo Erba Reagenti, Milano, Italy). Before each measurement, the pH meter was automatically calibrated for muscle temperature and using pH 4 and pH 7 buffered solutions (Crison, Lainate, Italy).

The colour of *Longissimus thoracis et lumborum* muscle samples was determined according to the CIE (Comission Internationale de l'Eclairage) colour system. A Minolta CR-300 colorimeter (light source D65; Minolta Camera Co. Ltd., Osaka, Japan) was used according to the method described by De Palo et al., 2015 [103]. Forty-eight hours after slaughtering, measurements were performed on fresh samples (L a b) and then on thawed samples (L* a* b*) in three different points. At each point, measurements were performed in triplicate, making a total of nine measurements per sample, according to the method described by De Palo et al., 2017 [104]. The colorimeter was calibrated

according to the Hunter-lab colour space system using a white title (L* = 99.2, a* = 1.0, b* = 1.9). The a* and b* values were used to determine chroma = $(a^2 + b^2)^{1/2}$ and hue (°) = tan−1(b/a) according to De Palo et al., 2012 [105]. Water holding capacity, thawing losses and cooking losses were measured as described by De Palo et al., 2014 [106]. The concentration of haem pigment was determined according to Hornsey, 1956 [107]. Results are presented as µg of acid haematin/g of muscle wet weight^{-1}.

3.7.1.2. Chemical composition

After thawing, samples of *Longissimus thoracis et lumborum* muscle were placed in an oven at 105°C until a constant weight was reached in order to determine moisture content. The protein content was measured according to ISO 937:1978. Intramuscular fat (IMF) was measured according to ISO 1443:1973. Each muscle was homogenised in a chloroform:ethanol solution (1:2, vol/vol) prior to the extraction of total lipids from IMF, performed using the method described by De Palo et al., 2016 [108]. Ash content was calculated according to ISO 936:1998.

3.7.1.3. Fatty acid profile

According to the methods described by De Palo et al., 2015 and 2016 [103,108], fatty acid methyl esters (FAME) were prepared by transesterification using methanol in the presence of 3% hydrochloric acid in methanol (vol/vol). FAME were determined using a Trace GC Thermo Quest Gas Chromatograph (Thermo Electron, Rodano, Milan, Italy) equipped with a flame ionization detector. The derivatives were separated on a capillary column (Supelco SP-2380 fused-silica column, 120 m length, 0.25 mm internal diameter and 0.20 mm film thickness). The injector and the detector temperatures were held at 260°C. Column oven program temperatures were as follows: T1 = 80°C, hold 1 min; T2 = 150°C, ramp at 15°C/min, hold 2 min; T3 = 220°C, ramp at 5°C/min, hold 2 min; T4 = 250°C, ramp at 15°C/min, hold 5 min. The flow rate of the carrier gas (He) was set at 0.8 mL/min. FAME identifications were based on the retention times of reference compounds (Sigma-Aldrich, St. Louis, MO, USA) and mass spectrometry. Fatty acid composition was expressed as the percentage of total FAME.
The amount of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n–3 and n–6 fatty acids, SFA/UFA, SFA/MUFA and SFA/PUFA were calculated to assess nutritional implications. Finally, atherogenic and thrombogenic indices were calculated according to the formulas provided by De Palo et al., 2017 [104]:

Atherogenic index (AI) = (C12:0 + 4 x C14:0 + C16:0) / [ΣMUFA + ΣPUFA (n–6) and (n–3)]

Thrombogenic index (TI) = (C14:0 + C16:0 + C:18) / [0.5ΣMUFA + 0.5ΣPUFA (n–6) + 3ΣPUFA(n–3) + (n–6)/ (n–3)]

3.7.2. Analysis of antioxidant enzymes and oxidation end-products

Plasma, liver and muscle samples were analysed for the following antioxidant enzymes: glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), according to the methods described by Tufarelli et al., 2016 [109] and Tateo et al., 2020 [110]. The following oxidation end-products were also determined in plasma and muscle samples: thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (HY), and dinitrophenylhydrazine (DNPH) as carbonylated proteins (PC), according to the methods described by De Palo et al., 2018 [111].

3.7.2.1. Analysis of thiobarbituric acid reactive substances (TBARs), protein carbonyls and hydroperoxides in plasma

Thiobarbituric acid reactive substances (TBARs) were measured fluorometrically according to Gondim et al. [112], by adding 100 mL plasma to a 0.37% thiobarbituric acid solution. Plasma reactive carbonyl derivative (RCD) levels were measured according to Faure and Lafond, 1995 [113]. RCD levels were determined by carbonyl reagent DNPH. Plasma (200 mL) was mixed with 1 mL water and 2 mL 20% trichloroacetic acid and centrifuged at 1000 × g for 10 min. The pellet was resuspended in 1 mL of 10 mmol/L DNPH and incubated for 60 min at 37.8°C. In the control condition, 1 mL of 1 mol/L hydrochloric acid was used instead of DNPH. Subsequently, 1 mL of 20% trichloroacetic acid was added, and the sample was centrifuged at 1000 × g for 10 min. The pellet was washed with 1:1 ethanolethyl acetate solution and centrifuged at 1000 × g for 10 min. The pellet was mixed with 1 mL of 6 mol/L guanidine (diluted in 20 mmol/L dihydrogenphosphate at pH 2.3). Finally, the sample was incubated for 40 min at 37.8°C. The absorbance was measured at 380nm. Hydroperoxides were analysed according to Södergren et al., 1998 [114]. Aliquots (90 mL) of plasma were transferred into eight microcentrifuge vials (1.5 mL). Ten microliters of 10 mM TPP in methanol were added to four of the vials to reduce ROOHs, thereby generating a quadruplicate of blanks. Methanol (10 mL) was added to the remaining four vials to produce a quadruplicate of test samples. All vials were then vortexed and incubated at room temperature for 30 min prior to the addition of 900 mL of FOX2 reagent. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2400 × g for 10 min with a swing-out rotor (Hettich Rotenta / RP centrifuge, Hettich-Zentrifuge, Tuttlingen, Germany). Absorbance of the supernatant was measured at 560 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). ROOH concentration in the plasma samples was calculated using the mean absorbance difference between quadruplicates of test samples and blank samples.

3.7.2.2. Analysis of thiobarbituric acid reactive substances (TBARs), protein carbonyls and hydroperoxides analyses in muscle

Minced muscle samples (5 g) were placed in a 50 mL test tube and homogenised with 15 mL deionised distilled water (DDW). Samples were treated as described by Maggiolino et al., 2020 [115]. The

concentration of TBARS were calculated by comparison against a standard curve constructed using 1,1,3,3-tetramethoxypropane, and the concentration of lipid oxidation was expressed as milligrams of malondialdehyde (MDA) per kg of meat. Two mL of homogenate (previously prepared for TBARS determination) were used for hydroperoxide quantification as described by De Palo et al., 2014 [116]. Results were expressed in micromoles per gram. Meat samples (2 g) were homogenised in 20 mL of 0.15 M KCl for 2 min and analysed for the quantification of protein carbonyls as described by De Palo et al., 2013 [117].

3.7.3. Statistical analysis

Data were statistically analysed using the software JMPpro v15 (SAS Institute Inc., Cary, NC). Each parameter was tested for normal distribution using the Shapiro Wilk test and normalized, when necessary, by box-cox transformation. A linear mixed effects model was constructed and the model fixed effects were the dietary treatment, the sex and their interaction. Then, each horse within sex and diet was considered as experimental unit and used as random variable for all analyses. The initial BW was set as a covariate for the slaughter BW model. Least squares means were separated using T-Student's adjusted *p*-values when at least a tendency F-test ($p \leq 0.10$) was detected in the fixed effect interaction term.

4. RESULTS

4.1. Welfare assessment

The results described in sections 4.2. and 4.3. are adapted from Raspa et al., 2020a [75] and Raspa et al., 2020b [71], respectively.

4.2. Welfare assessment: stocking density, feeding management and welfare indicators

A total of 561 horses were evaluated. The horses belonged to Italian or French heavy draft breeds, and the mean age (± SD) was 16 (± 8) months.

The median values (plus 25th–75th percentiles) for environment temperature (°C) and relative humidity (%) over the seven surveys were 13 °C (11–23 °C) and 73% (55–75%), respectively.

The Cohen's kappa coefficients (Ks) for inter-observer reliability ranged between 0.61 and 1, indicating substantial (K = 0.61–0.80) to strong (K = 0.80–1) agreement between the expert evaluators.

4.2.1. Results considering the median cut-off value for the stocking density

Table 8 shows the results of the Mann–Whitney U-test and Fisher's exact tests. The median cut-off value for the stocking density was calculated in order to divide and compare the survey data according to whether the horses were housed at a low stocking density (LSD^{50th}) or a high stocking density (HSD^{50th}). The median cut-off value for the stocking density (m^2/horse) was 3.95 m^2/horse (LSD^{50th} group ≥3.95 m^2/horse vs. HSD^{50th} group <3.95 m^2/horse).

When the two groups were compared on the basis of the median stocking density cut-off value, significant differences were found in two of the welfare indicators of good feeding: the space at the feed bunk (m/horse; $p<0.001$) and the BCS ($p=0.004$). The ideal feeding space per horses at a feed trough is reported to be 1 m/horse [51]; the median space (plus 25th–75th percentiles) revealed here was 0.95 (0.70–1.30) m/horse for the LSD^{50th} group and 0.6 (0.42–0.79) m/horse for the HSD^{50th} group. Moreover, the median number of horses within the group pens scored as thin was higher for the horses in the HSD^{50th} group at 0.5 (0–2.25) compared with 0 (0–0) for the LSD^{50th} group.

Considering the welfare principle of good housing, the welfare indicators "coat cleanliness" and "bedding quantity" were shown to be influenced by the stocking density. The median number of animals scored as having a dirty coat (coat cleanliness score of 1 to 3) was lower (3, 1–4) in the LSD^{50th} group than in the HSD^{50th} group (5, 2–7) ($p=0.004$). Therefore, a higher stocking density was associated with a significantly higher number of horses scored as having a dirty coat. The frequency (%) of pens scored as having an inadequate quantity of bedding was 56.8% in the LSD^{50th} group and 83.3% in the HSD^{50th} group ($p=0.021$), revealing that when horses were housed at higher densities, a significantly higher percentage of pens had inadequate amounts of bedding material covering the pen floor.

For the welfare principle of good health, just one welfare indicator was affected by stocking density: the median number of horses with a cough was significantly lower in the LSD^{50th} group than in the HSD^{50th} group (p=0.028).

Finally, with regard to the welfare principle of appropriate behaviour, two indicators were affected by stocking density: feeding behaviour and resting in a standing position. The median number of horses exhibiting feeding behaviour at the moment of the observation was significantly higher in the HSD^{50th} group (5, 2–6.75) than the LSD^{50th} group (2, 0.5–4) (p=0.001). This suggests that, on the farm in question, horses housed at a higher stocking density are more likely to express feeding behaviour. Moreover, with regard to resting in standing position, more animals were found to express this behavior in the HSD^{50th} group (1, 0–3) than LSD^{50th} (0, 0–2) (p=0.012).

Table 8. Statistical analysis performed using the median cut-off value for the stocking density (3.95 m^2/horse). Nondichotomous variables are expressed as the median number of horses (plus 25th–75th percentiles) within pens that show a specific score or health condition or are performing a specific behaviour. Space at the feed bunk is expressed as the median (plus 25th–75th percentiles) length in metres available per horse. Nondichotomous variables were analysed using the Mann–Whitney U test: the test statistic (U) and p-values are reported. Dichotomous variables are expressed as frequencies (%) and were analysed using the Fisher exact test: the test statistic (χ^2) and p-values are reported. Data were considered significant for p-values <0.05. Adapted from Raspa et al., 2020a [75].

Welfare Principle	Welfare Indicator	LSD^{50th} median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 37) with ≥3.95 m^2/horse	HSD^{50th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 36) with <3.95 m^2/horse	Test Statistics § Mann–Whitney U Test (U) Fisher Exact Test (χ^2)	*p*-values
Good feeding	Space at feed bunk (m/horse)	0.95 (0.70–1.30)	0.6 (0.42–0.79)	U = 194.00	<0.001*
	BCS [0]	0 (0–0)	0.5 (0–2.25)	U = 459.00	0.004*
	Water point cleanliness [a]	Adequate: 68.6% Inadequate: 31.4%	Adequate: 63.9% Inadequate: 36.1%	χ^2 = 0.174	0.803
Good housing	Coat cleanliness [1]	3 (1–4)	5 (2–7)	U = 408.50	0.004*
	Bedding cleanliness [a]	Adequate: 22.9% Inadequate: 77.1%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 0.387	0.757
	Bedding quantity [a]	Adequate: 43.2% Inadequate: 56.8%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 6.121	0.021*
Good health	Skin lesions [2]	1 (0.5–2)	1 (0–2)	U = 658.50	0.931
	Mane condition [3]	4 (3–7)	5.5 (3–9.5)	U = 389.00	0.142
	Tail condition [4]	1 (0–1.5)	1.5 (0–4)	U = 470.00	0.056
	Swollen joints [5]	0 (0–1)	1 (0–2)	U = 602.00	0.444
	State of awareness [6]	0 (0–0)	0 (0–0)	U = 610.50	0.075
	Abnormal breathing [7]	1 (0–1)	0 (0–0.75)	U = 631.50	0.626
	Nasal discharges [8]	0 (0–2)	0 (0–1)	U = 574.00	0.249
	Ocular discharges [9]	0 (0–1)	0 (0–1)	U = 650.00	0.833
	Consistency of faeces [a]	Adequate: 0% Inadequate: 100%	Adequate: 8.3% Inadequate: 91.7%	χ^2 = 3.215	0.115
	Cough [a]	0 (0–0)	0 (0–1)	U = 522.00	0.028 *

Appropriate behaviour	Feeding [10]	2 (0.5–4)	5 (2–6.75)	U = 353.50	0.001 *
	Watching [11]	1 (0–3)	1.5 (0–4)	U = 598.00	0.442
	Mutual grooming [12]	0 (0–0)	0 (0–0)	U = 648.50	0.574
	Resting in a standing position [13]	0 (0–2)	1 (0–3)	U = 452.00	0.012 *
	Resting in a lying position [14]	0 (0–0)	0 (0–1)	U = 597.50	0.306
	Playing [15]	0 (0–0)	0 (0–0)	U = 623.00	0.574
	Sexual behavior [16]	0 (0–0)	0 (0–0)	U = 646.50	0.532
	Aggressive behavior [17]	0 (0–0)	0 (0–0)	U = 566.00	0.076
	Stereotypic behavior [18]	0 (0–0)	0 (0–0)	U = 666.00	1

* Significant values. [§] The degrees of freedom for each analysed variable were equal to 1. [a] Dichotomous variables expressed as frequencies (%) of occurrence within the multiple pens. [0] N of horse scored as thin using the specifically developed 3-point scoring system. [1] N of horses with a coat cleanliness score of 1, 2 or 3, using the specifically developed 5-point scoring system. [2] N of horses within the pens presenting skin lesions, including areas of alopecia, injuries, tumefaction, or swelling. [3] N of horses presenting a ruined mane, as defined by a score of 3, using the specifically developed 3-point scoring system. [4] N of horses presenting a ruined tail, as defined by a score of 3, using the specifically developed 3-point scoring system. [5] N of horses presenting swollen joints. [6] N of horses presenting an abnormal state of awareness. [7] N of horses presenting abnormal breathing. [8] N of horses presenting nasal discharges. [9] N of horses presenting ocular discharges. [10] N of horses feeding. [11] N of horses watching. [12] N of horses engaged in mutual grooming. [13] N of horses resting in a standing position. [14] N of horses resting in a lying position. [15] N of horses playing. [16] N of horses performing sexual behaviours. [17] N of horses engaged in aggressive behaviours. [18] N of horses performing stereotypic behaviours.

4.2.2. Results considering the 75th percentile cut-off value for the stocking density

The data were re-analysed by considering a stocking density (m^2/horse) cut-off value equal to the 75th percentile: 4.75 m^2/horse. This analysis was performed to assess whether a small increase in space allowance per horse would lead to any improvements in horse welfare. Therefore, animals in the LSD^{75th} group had a space allowance ≥4.75 m^2/horse, whereas those in the HSD^{75th} group had <4.75 m^2/horse. The results of the Mann–Whitney U test and the Fisher exact tests are shown in Table 9.

Considering the welfare principle of good feeding, once again, a significant difference was shown in relation to space at the feed bunk (m/horse; $p<0.001$). When we consider a lower stocking density, this automatically correlates with a larger feeding space per animal at the feed bunk. In fact, the median space at the feed bunk was 1.3 (1.10–1.54) m/horse in the LSD^{75th} group vs. 0.70 (0.45–0.84) m/horse for the HSD^{75th} group.

Moving on to the welfare principle of good housing, the data regarding coat cleanliness and the bedding quantity were again found to differ significantly between the low and high stocking density groups. The median number of animals scored to have a dirty coat (cleanliness score of 1 to 3) was higher (4, 2–7) in the HSD^{75th} group than in the LSD^{75th} group (2, 1–4) ($p = 0.005$). The frequency (%) of pens scored as having an inadequate quantity of bedding was significantly lower (44.48%) in the LSD^{75th} group compared with the HSD^{75th} group (78.2%) ($p=0.016$).

For the welfare principle of good health, both mane condition and tail condition were significantly influenced by stocking density when defining the groups by the 75th percentile cut-off, with p-values of 0.038 and 0.024, respectively. The median number of horses presenting a ruined mane (score of 3) was significantly higher (5, 3–8) in the HSD^{75th} group than the LSD^{75th} group (3.5, 3–4.75) ($p = 0.038$). Moreover, the median number of horses presenting a ruined tail (score of 3) was lower (0, 0–1) in the LSD^{75th} group than in the HSD^{75th} group (1, 0–3) ($p=0.024$).

Considering the welfare principle of appropriate behaviour, the median number of horses expressing feeding behaviour at the moment of the welfare assessment was higher in the HSD^{75th} group (3, 2–6) than in the LSD^{75th} group (1.5, 0–3.25) ($p=0.002$). A significant difference between groups was also found for the median number of horses standing in a resting position, which was higher in the HSD^{75th} group (1, 0–3) than the LSD^{75th} group (0, 0–0.25) ($p=0.003$).

Interestingly, in contrast with the previous statistical analysis in which the median stocking density was used as the cut-off value, no statistical significance was shown for BCS or the presence of a cough when groups were compared on the basis of the 75th percentile cut-off value.

Table 9. Statistical analysis performed using the 75th percentile cut-off value (4.75 m^2/horse). Nondichotomous variables are expressed as the median number of horses (plus 25th–75th percentiles) presenting a specific score or health condition or performing a specific behaviour. Space at the feed bunk is expressed as median (plus 25th–75th percentiles) length in metres available per horse. Nondichotomous variables were analysed using the Mann–Whitney U test: test statistic (U) and p-values are reported. Dichotomous variables are expressed as frequencies (%) and were analysed using the Fisher exact test: the test statistic (χ^2), degrees of freedom and p-values are reported. Data were considered significant for p-values <0.05. Adapted from Raspa et al., 2020a [75].

Welfare Principle	Welfare Indicator	LSD^{75th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 18) with ≥4.75 m^2/horse	HSD^{75th} median values (25th–75th percentiles) and frequencies (%) for groups (n = 55) with <4.75 m^2/horse	Test Statistics § Mann–Whitney U test (U) Fisher Exact Test (χ^2)	p-values
Good feeding	Space at feed bunk (m/horse)	1.3 (1.10–1.54)	0.70 (0.45–0.84)	U = 95.00	<0.001*
	BCS [0]	0 (0–0)	0 (0–2)	U = 388.00	0.105
	Water point cleanliness [a]	Clean (0): 62.5% Dirty (1): 37.5%	Clean (0): 67.3% Dirty (1): 32.7%	χ^2 = 0.126 (1)	0.769
Good housing	Coat cleanliness [1]	2 (1–4)	4 (2–7)	U = 275.50	0.005*
	Bedding cleanliness [a]	Adequate: 29.4% Inadequate: 70.61%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 1.275 (1)	0.299
	Bedding quantity [a]	Adequate: 55.6% Inadequate: 44.4%	Adequate: 21.8% Inadequate: 78.2%	χ^2 = 7.331 (1)	0.016*
Good health	Skin lesions [2]	1 (0–2)	1 (0–2)	U = 443.50	0.49
	Mane condition [3]	3.5 (3–4.75)	5 (3–8)	U = 245.50	0.038*
	Tail condition [4]	0 (0–1)	1 (0–3)	U = 313.50	0.024*
	Swollen joints [5]	0 (0–1)	1 (0–2)	U = 374.50	0.095
	State of awareness [6]	0 (0–0)	0 (0–0)	U = 468.00	0.315
	Abnormal breathing [7]	0 (0–1)	0 (0–1)	U = 494.00	0.095
	Nasal discharges [8]	0 (0–2)	0 (0–1)	U = 484.00	0.873
	Ocular discharges [9]	0 (0–0)	0 (0–1)	U = 391.00	0.113
	Consistency of faeces [a]	Adequate: 0% Inadequate: 100%	Adequate: 5.5% Inadequate: 94.5%	χ^2 = 1.024 (1)	0.570
	Cough [a]	0 (0–0)	0 (0–0.5)	U = 420.00	0.183

	Feeding [10]	1.5 (0–3.25)	3 (2–6)	U = 260.50	0.002*
	Watching [11]	1.5 (0–2.25)	1 (0–4)	U = 413.00	0.282
	Mutual grooming [12]	0 (0–0)	0 (0–0)	U = 449.00	0.087
	Resting in a standing position [13]	0 (0–0.25)	1 (0–3)	U = 277.50	0.003*
Appropriate behaviour	Resting in a lying position [14]	0 (0–0.25)	0 (0–0)	U = 489.00	0.917
	Playing [15]	0 (0–0)	0 (0–0)	U = 418.00	0.125
	Sexual behavior [16]	0 (0–0)	0 (0–0)	U = 468.00	0.315
	Aggressive behavior [17]	0 (0–0)	0 (0–0)	U = 396.00	0.120
	Stereotypic behavior [18]	0 (0–0)	0 (0–0)	U = 495.00	1

* Significant values. § The degrees of freedoms for each analysed variable were equal to 1. [a] Dichotomous variables expressed as frequencies (%) of occurrence within the multiple pens. [0] N of horses scored as thin using the specifically developed 3-point scoring system. [1] N of horses with a coat cleanliness score of 1, 2 or 3, using the specifically developed 5-point scoring system. [2] N of horses within the pens presenting skin lesions, including areas of alopecia, injuries, tumefaction, or swelling. [3] N of horses presenting a ruined mane, as defined by a score of 3, using the specifically developed 3-point scoring system. [4] N of horses presenting a ruined tail, as defined by a score of 3, using the specifically developed 3-point scoring system. [5] N of horses presenting swollen joints. [6] N of horses presenting an abnormal state of awareness. [7] N of horses presenting abnormal breathing. [8] N of horses presenting nasal discharges. [9] N of horses presenting ocular discharges. [10] N of horses feeding. [11] N of horses watching. [12] N of horses engaged in mutual grooming. [13] N of horses resting in a standing position. [14] N of horses resting in a lying position. [15] N of horses playing. [16] N of horses performing sexual behaviours. [17] N of horses engaged in aggressive behaviours. [18] N of horses performing stereotypic behaviours.

4.3. Welfare assessment: stocking density and behavioural activities

The inter-observer reliability was high: K = 0.83 (95% CI [0.72–0.94]) The intra-observer reliability was substantial K = 0.67 (95% CI [0.59–0.75]) for the first evaluator, and very high for the second evaluator K = 0.81 (95% CI [0.75–0.87]) [118].

A total amount of 96 scans per horse were performed each day, providing a total of 6,336 scans sampled over the 72 h video-recordings.

4.3.1. Correlations between time-budget and stocking densities within group pens

The reduction in the stocking density and the subsequent increase in the space allowance per horse (from 4 to 6 m^2/horse) was positively correlated with locomotion (r=0.89, p=0.001), playing (r=0.73, p=0.024), and self-grooming (r=0.76, p=0.018) (Table 10). The data obtained revealed that the reduction in stocking density correlated with a higher frequency in the expression of these activities by horses. Locomotion showed a positive correlation with the reduction in stocking density during both the 12 daylight hours (%/12 light hours) (r=0.76, p=0.017) and 12 night hours (%/12 night hours) (r=0.67, p=0.049). Playing seemed to be positively and significantly correlated with the reduction in stocking density during the 12 daylight hours (r=0.79, p=0.012), but not during the 12 night hours (r=0.29, p=0.444); the same was true for self-grooming, which showed a positive correlation during the 12 daylight hours (r=0.78, p=0.014), but not during the 12 night hours (r=0.48, p=0.193).

Although standing was not significantly correlated with stocking density over the whole 24 h period, a negative correlation was shown during the 12 night hours (r=−0.68, p=0.049). Based on this data, the reduction in the stocking density was associated with a reduction in the expression of standing behaviour during the 12 night hours of the 24 h period.

Table 10. Associations between the time-budgets (%/24 h; %/12 light hours; %/12 night hours) and stocking densities among the group pens. Adapted from Raspa et al., 2020b [71].

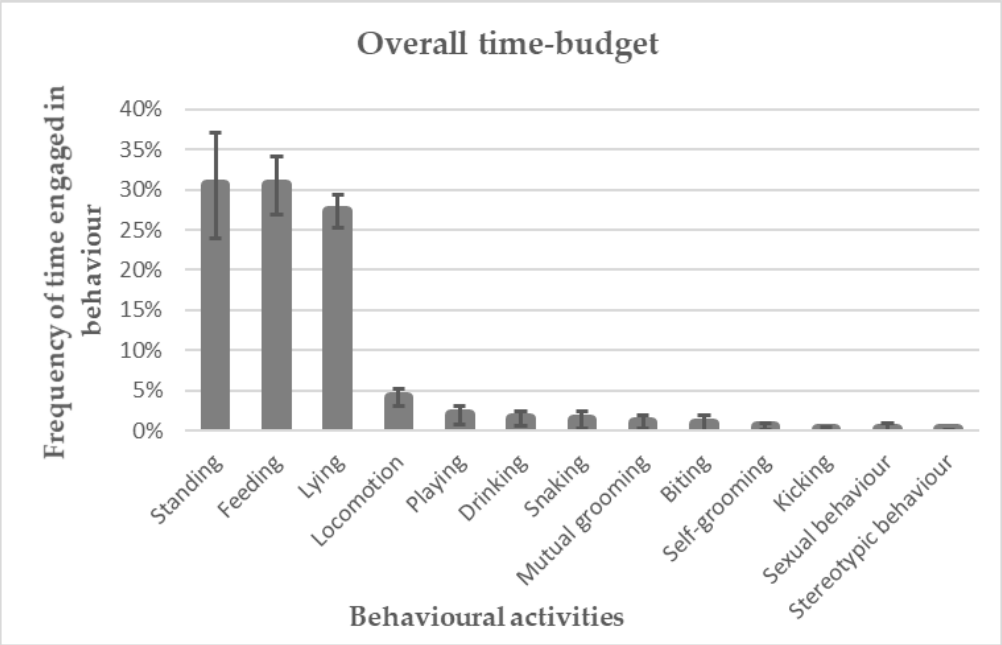
Behavioural Activities	Stocking Density					
	%/24 h		%/12 Light Hours		%/12 Dark Hours	
	r [a]	*p*-value	*r* [a]	*p*-value	*r* [a]	*p*-value
Standing	−0.61	0.079	−0.51	0.157	−0.68	0.049 *
Feeding	−0.23	0.559	−0.14	0.724	−0.32	0.396
Lying	0.59	0.094	−0.08	0.839	0.57	0.112
Locomotion	0.89	0.001 *	0.76	0.017 *	0.67	0.049 *
Playing	0.73	0.024 *	0.79	0.012 *	0.29	0.444
Drinking	−0.29	0.450	−0.56	0.114	0.00	0.997
Snaking	0.23	0.553	0.28	0.461	0.04	0.911
Mutual grooming	0.29	0.449	0.28	0.473	0.17	0.659
Biting	0.36	0.346	0.29	0.450	0.39	0.301
Self-grooming	0.76	0.018 *	0.78	0.014 *	0.48	0.193
Kicking	0.35	0.361	0.37	0.330	0.10	0.807
Sexual behaviour	0.38	0.317	0.39	0.297	0.00	1.000
Stereotypic behaviour	0.43	0.244	0.43	0.244	0.43	0.244

[a] Pearson's correlation coefficient. * Statistical significance $p<0.05$

4.3.2. Overall time-budget and time frame

Figure 8 shows the overall time-budget of each behavioural activity engaged in by horses reared for meat production. The main expressed behavioural activities were: standing (30.56% ± 6.56%), feeding (30.55% ± 3.59%), and lying (27.33% ± 2.05%). Locomotion occupied only 4.07% ± 1.06% of the time. All the other activities occupied less than the 2% of the overall time-budget. In particular, stereotypic behaviours were performed the least, occupying just 0.04% ± 0.12% of the time.

Figure 8. Frequency of time (%) spent by horses in behavioural activities. Adapted from Raspa et al., 2020b [71].



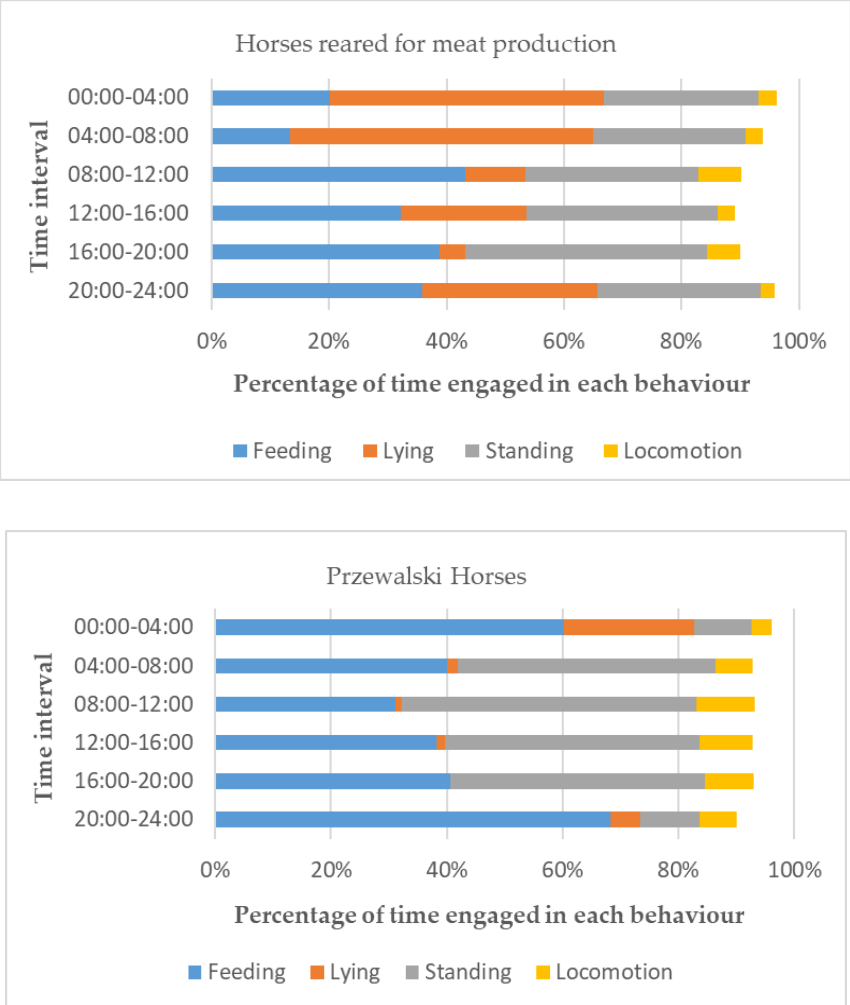
The overall time-budget of each behavioural activity shown by horses was divided into six time intervals (00:00–04:00; 04:00–08:00; 08:00–12:00; 12:00–16:00; 16:00–20:00; 20:00–24:00). As reported in Table 11, the main activity from 00:00–04:00 was lying (46.61% ± 1.19%), followed by standing (26.33% ± 4.05%), feeding (20.14% ± 2.12%), and locomotion (3.07% ± 1.63%). The time interval 04:00–08:00 showed a similar pattern, with lying being the main behaviour (51.48% ± 6.79%), followed by standing (26.01 ± 4.31%), feeding (13.43% ± 4.96 %), and locomotion (3.01% ± 0.75%). Considering the 08:00–12:00 time interval, the main activity was feeding (43.11% ± 3.65%), followed by standing (29.40% ± 6.99%), lying (10.30% ± 5.10%), and locomotion (7.38% ± 4.66%). The main activity expressed during the 12:00–16:00 time interval was standing (32.67% ± 6.93%), then feeding (31.94% ± 3.40%), lying (21.38% ± 0.93%), and locomotion (2.95% ± 0.15%). The same pattern of expression was also shown for 16:00 to 20:00, where the main expressed activity was standing (41.06% ± 1.48%), followed by feeding (38.74% ± 5.64%), locomotion (5.70% ± 4.26%), and lying (4.46% ± 2.13%). From 20:00 to 24:00, feeding was the main activity (35.94% ± 4.19%), followed by lying (29.77% ± 2.61%), standing (27.86% ± 6.64%), and locomotion (2.34% ± 1.71%).
Stereotypic behaviour was only present during the time intervals 12:00 to 16:00 and 20:00 to 24:00, although horses were only engaged in this activity for 0.12% ± 0.20% of the time.

Table 11. Overall time-budget and time frames of different behavioural activities performed by horses reared for meat production. Frequencies (%) of behavioural activities are expressed as means ± SD. Adapted from Raspa et al., 2020b [71].

Behavioural Activities (%)	Overall Time-Budget	00:00–04:00	04:00–08:00	08:00–12:00	12:00–16:00	16:00–20:00	20:00–24:00
Standing	30.56 ± 6.56	26.33 ± 4.05	26.01 ± 4.31	29.40 ± 6.99	32.67 ± 6.93	41.06 ± 1.48	27.86 ± 6.64
Feeding	30.55 ± 3.59	20.14 ± 2.12	13.43 ± 4.96	43.11 ± 3.65	31.94 ± 3.40	38.74 ± 5.64	35.94 ± 4.19
Lying	27.33 ± 2.05	46.61 ± 1.19	51.48 ± 6.79	10.30 ± 5.10	21.38 ± 0.93	4.46 ± 2.13	29.77 ± 2.61
Locomotion	4.07 ± 1.06	3.07 ± 1.63	3.01 ± 0.75	7.38 ± 4.66	2.95 ± 0.15	5.70 ± 4.26	2.34 ± 1.71
Playing	1.97 ± 1.16	0.58 ± 1.00	1.56 ± 0.90	3.36 ± 3.03	3.13 ± 1.04.	3.04 ± 2.12	0.17 ± 0.30
Drinking	1.51 ± 0.86	1.22 ± 0.91	1.19 ± 0.58	1.59 ± 1.18	2.03 ± 1.10	0.90 ± 0.62	2.17 ± 0.66
Snaking	1.27 ± 1.07	0.43 ± 0.54	1.33 ± 0.99	2.08 ± 0.90	1.24 ± 1.04	2.11 ± 1.72	0.43 ± 0.54
Mutual grooming	1.07 ± 0.85	0.69 ± 1.20	1.04 ± 0.90	1.01 ± 0.95	1.74 ± 1.59	1.56 ± 1.38	0.38 ± 0.39
Biting	0.84 ± 1.00	0.43 ± 0.54	0.78 ± 1.14	0.81 ± 0.56	1.22 ± 1.08	1.50 ± 1.12	0.29 ± 0.27
Self-grooming	0.52 ± 0.37	0.49 ± 0.43	0.17 ± 0.30	0.64 ± 0.70	0.93 ± 0.70	0.49 ± 0.22	0.41 ± 0.36
Kicking	0.19 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.18	0.55 ± 0.74	0.26 ± 0.26	0.12 ± 0.20
Sexual behaviour	0.07 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.20	0.12 ± 0.20	0.17 ± 0.15	0.00 ± 0.00
Stereotypic behaviour	0.04 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.20	0.00 ± 0.00	0.12 ± 0.20

Figure 9 shows the comparison of the 24 h time frame of the main expressed behavioural activities (standing, feeding, lying, and locomotion) performed by the horses reared for meat production and young Przewalski horses (data adapted from Boyd et al., 1988 [94]).

Figure 9. Comparison of the 24 h time frame of the main expressed behavioural activities (standing, feeding, lying, and locomotion) engaged in by the horses reared for meat production and wild-living Przewalski horses (data adapted from Boyd et al., 1988 [94]). Adapted from Raspa et al., 2020b [71].



4.4. Feeding management: gut health, behaviour, production performances

4.5. Feeding management: gut health

4.5.1. Scoring of the gastric mucosa lesions

The scores of the gastric mucosa lesions are summarized in Table 12. The glandular region of horses in HS group presented gastric mucosa lesions significantly more severe compared to that seen in horses belonging to the HF group (p=0.01). Moreover, a statistical tendency (p=0.06) was found for the squamous region which resulted characterised by higher score in HS than HF according to the diet.

Regarding sex or interaction between diet and sex, it did not influence the severity of gastric lesions both in the glandular and squamous regions (p>0.05).

Table 12. Scoring of gastric mucosa of the glandular and squamous regions according to the two dietary treatments (HS vs. HF), sex and their interaction. Data are expressed as mean (± standard deviation, SD).

					p-value		
	HS	HF	Female	Male	Diet	Sex	Diet*Sex
Glandular region	2.17 (1.17)	0.407 (0.69)	1.23 (1.13)	1.25 (1.58)	0.01*	0.45	0.98
Squamous region	1.56 (0.73)	0.806 (1.13)	1.096 (1.04)	1.256 (1.03)	0.06	0.96	0.32

HS=high starch; HF=high fibre; * Statistical significance p<0.05

4.5.2. Dry matter, Organic matter and Ash content

The percentage of dry matter (DM) and of organic matter (OM) and ash content (as a percentage of DM) in samples obtained from the different intestinal compartments of horses are shown in Table 12 according to the dietary treatment received (HS vs. HF). The statistical analyses for the effect of diet and sex and their interaction are also shown. The DM content in the RDC was significantly affected by dietary treatment (HS vs. HF). Specifically, horses fed the HS diet showed a higher DM content in the RCD compared with horses fed the HF diet (p<0.01). The OM content was significantly higher in SF, PF, RDC and RE in the horses fed the HF diet compared with those fed the HS diet (p<0.01), and a significant diet*sex interaction was found for DM content in the SF (p=0.02). Moreover, the ash content was significantly higher in SF, PF, RDC and RE in the horses fed HS diet compared with horses fed the HF diet (p<0.01). Once again, a significant diet*sex interaction was identified in relation to the SF (p=0.04).

Table 13. Comparison between DM, OM and Ash content according to the dietary treatments (HS vs. HF). Data not normally distributed are expressed as medians (25^{th}–75^{th} percentiles); data normally distributed are expressed as means (SEM).

		HS	HF	*p*-value		
				Diet	Sex	Diet*Sex
DM (%)	SI	3.94 (0.38)	4.49 (0.40)	0.39	0.13	0.17
	CAE	5.33 (0.56)	5.86 (0.35)	0.33	0.75	0.43
	SF	11.51 (0.50)	11.19 (0.36)	0.86	0.94	0.27
	PF	11.60 (1.09)	9.83 (0.41)	0.23	0.84	0.58
	RDC	14.17 (0.28)	11.04 (0.56)	<0.01*	0.84	0.25
	RE	18.18 (0.61)	16.16 (1.30)	0.31	0.09	0.46
OM (%DM)	SI	n.a.	n.a			
	CAE	79.42 (1.29)	82.47 (1.06)	0.11	0.71	0.95
	SF	86.25 (84.64–87.59)	88.32 (87.98–88.84)	<0.01*	0.19	0.02*[a]
	PF	82.63 (0.69)	86.66 (0.38)	<0.01*	0.47	0.29
	RDC	82.48 (0.74)	86.83 (0.38)	<0.01*	0.87	0.82
	RE	82.95 (0.82)	88.80 (0.46)	<0.01*	0.75	0.61
Ash (%DM)	SI	n.a.	n.a			
	CAE	20.57 (1.29)	17.52 (1.06)	0.11	0.71	0.95
	SF	13.74 (12.40–15.35)	11.67 (11.15–12.01)	<0.01*	0.31	0.04*[b]
	PF	17.36 (0.69)	13.33 (0.38)	<0.01*	0.47	0.29
	RDC	17.51 (0.74)	13.16 (0.38)	<0.01*	0.87	0.82
	RE	17.04 (0.82)	11.19 (0.46)	<0.01*	0.75	0.61

HS=high starch; HF=high fibre; SI=small intestine, n.a.=not analysed; CAE=apex of the caecum; SF=ventral diaphragmatic flexure of the colon; PF= pelvic flexure; RDC=right dorsal colon; RE=rectum. *statistical significance $p<0.05$.

*[a] females HF 88.48 (88.08–88.84)^{A}; males HF 88.16 (85.96–89.15)^{A}; males HS 87.60 (86.20–87.74)^{A}; females HS 85.49 (83.46–86.36)^{B}. ^{A,B} $p<0.05$.

*[b] females HS 14.51 (13.65–16.54)^{A}; males HS 12.41 (12.27–13.80)^{B}; males HF 11.84 (10.85–14.04)^{BC}; females HF 11.52 (11.16–11.92)^{C}. ^{A,B,C} $p<0.05$.

4.5.3. Particle size distribution

The results of the particle size analysis obtained by wet sieving faecal samples from each of the nominated intestinal compartments are summarised in Table 13. Results are shown according to the dietary treatment received.

In the CAE, dietary treatment significantly affected the particle size distribution. In particular, the proportion of faecal particles retained on the 2 mm sieve was significantly higher in horses receiving the HF diet compared with those receiving the HS diet (p<0.01). Instead, the fraction of particles that washed through the finest sieve (<1 mm) was higher in the HS group than in the HF group (p=0.02).
With regard to the SF compartment, differences were found between the two groups related to the proportion of particles retained on the 8 mm sieve, which was higher in horses fed HS diet compared with those on the HF diet (p<0.01). Once again, the proportion of particles retained by the 2 mm sieve was higher in the in HF group compared with the HS group (p=0.01). In this case, a significant diet*sex interaction was also present (p=0.05). Finally, a higher fraction of particles washed through the finest sieve (<1 mm) in samples from the HS group compared with samples from the HF group (p=0.01).
In the PF, similar to the previous compartment along the digestive tract, the SF, the proportion of faecal particles retained on 8 mm sieve was greater in horses receiving the HS diet compared with those on the HF diet (p=0.03). Moreover, in the PF, dietary treatment (HS vs. HF) also had a significant effect on the proportion of particles retained on the 4 mm sieve (p=0.05). In this case, the proportion of faecal particles was higher in horses fed the HF diet than in those receiving the HS diet, and a significant diet*sex interaction (p=0.05) was shown. Once again, the proportion of faecal particles retained on 2 mm sieve in the PF was higher in horses fed the HF diet than those fed the HS diet (p<0.01); whereas the fraction that washed through the finest sieve (<1 mm) was higher in horses in the HS group (p=0.03).
In the RDC, a significant difference in the fraction of particle sizes retained was observed for the 8 mm sieve, which was higher for faecal samples collected from horses in the HS group (p<0.01).
In the RE, dietary treatment once again significantly affected the proportion of particles retained on the 8 mm sieve (p=0.02). The proportion of particles was higher in horses fed the HS diet than those fed the HF diet. As in the PF, the opposite was true for the 4 mm sieve, for which the proportion of faecal particles retained was greater in horses fed HF diet than in those fed the HS diet (p<0.01. The fraction that washed through the finest sieve (<1 mm) was once again higher in case of horses fed the HS diet (p<0.01), as occurred in all the other intestinal compartments with the exception of the RDC.

Table 14. Comparison of particle size distributions according to the diet (HS vs. HF). Values are expressed as a percentage (%) of particles, on a dry matter basis, retained by each sieve (8, 4, 2, 1 and <1 mm). Data not normally distributed are expressed as medians (25^{th}–75^{th} percentiles); data normally distributed are expressed as means (SEM).

		HS	HF	*p*-value		
				Diet	Sex	Diet*Sex
SI		n.a.	n.a.	-	-	-
CAE	8 mm	3.41 (2.91–8.58)	2.76 (1.17–4.06)	0.09	0.35	0.70
	4 mm	11.14 (7.39–14.48)	21.15 (10.53–27.93)	0.06	0.89	0.30
	2 mm	7.75 (0.88)	14.31 (1.39)	<0.01*	0.20	0.94
	1 mm	6.34 (0.65)	7.46 (0.82)	0.17	0.22	0.33
	<1 mm	69 50 (2.83)	54.33 (3.92)	0.02*	0.87	0.49
SF	8 mm	4.68 (0.37)	1.86 (0.29)	<0.01*	0.52	0.52
	4 mm	18.73 (2.39)	23.71 (2.43)	0.24	0.77	0.58
	2 mm	11.55 (1.69)	17.92 (2.07)	0.01*	0.54	0.05*[a]
	1 mm	7.48 (0.57)	8.22 (0.64)	0.42	0.82	0.71
	<1 mm	57.54 (2.92)	48.27 (1.40)	0.01*	0.90	0.23
PF	8 mm	3.65 (2.31–6.27)	1.34 (1.09–2.47)	0.03*	0.76	0.31
	4 mm	17.85 (1.92)	25.95 (2.64)	0.05*	0.49	0.05*[b]
	2 mm	10.58 (1.21)	15.78 (0.78)	<0.01*	0.39	0.34
	1 mm	7.31 (5.12–11.13)	6.39 (5.83–6.95)	0.75	0.87	0.48
	<1 mm	59.21 (3.38)	48.97 (1.94)	0.03*	0.52	0.33
RDC	8 mm	4.54 (2.17–5.98)	1.65 (1.01–2.30)	<0.01*	0.18	0.96
	4 mm	21.70 (1.93)	25.67 (2.14)	0.20	0.26	0.62
	2 mm	1.75 (1.55)	18.14 (1.64)	0.06	0.57	0.85
	1 mm	6.64 (4.94–8.97)	6.78 (6.42–7.46)	0.82	0.75	0.91
	<1 mm	54.03 (3.37)	47.30 (1.57)	0.12	0.56	0.60
RE	8 mm	3.75 (0.52)	2.13 (0.27)	0.02*	0.08	0.85
	4 mm	19.28 (1.81)	33.13 (2.76)	<0.01*	0.67	0.16
	2 mm	12.92 (1.21)	17.90 (2.21)	0.07	0.50	0.49
	1 mm	10.29 (1.31)	9.20 (1.76)	0.56	0.41	0.86
	<1 mm	53.72 (2.29)	37.61 (1.91)	<0.01*	0.68	0.27

HS=high starch; HF=high fibre; SI=small intestine, n.a.=not analysed according to methods section; CAE=apex of the caecum; SF=ventral diaphragmatic flexure of the colon; PF= pelvic flexure; RDC=right dorsal colon; RE=rectum; *statistical significance $p<0.05$.

*[a] males HF 21.2 (13.27–27.67)^{A}; females HF 17.72 (14.35–21.82)^{A}; females HS 14.84 (12.08–17.46)^{AB}; males HS 8.5 (3.48–10.55)^{B}. ^{A,B} $p<0.05$.

*[b] females HF 26.04 (18.16–37.86)^{A}; males HS 21.72 (19.55–27.58)^{AB}; males HF 22.22 (17.81–28.07)^{AB}; females HS 14.73 (11.94–15.14)^{B}. ^{A,B} $p<0.05$.

4.5.4. Volatile fatty acids (VFAs)

Table 14 reports the results of the volatile fatty acid analysis conducted on samples obtained from the distinct intestinal compartments of horses receiving the two dietary treatments. The total amounts of VFAs (mg/100 ml) produced in the in the SF, PF, RDC and RE were significantly higher in horses receiving the HS diet compared with those receiving the HF diet ($p<0.01$); no differences were found in total VFAs between treatment groups for the SI and CAE. Moreover, the percentage (%) of valeric acid on the total VFAs was significantly higher ($p<0.01$) in horses receiving the HS diet for all the sampled gut compartments – CAE, SF, PF, RDC and RE; conversely, the valeric acid was not detected in the HF group.

In the CAE, a significantly higher production (%) of acetic acid, propionic acid, iso-butyric acid and butyric acid was detected in the horses fed the HF diet compared with horses fed the HS diet ($p<0.01$).

In the SF, a significantly higher production (%) of acetic acid, propionic acid and butyric acid was observed in horses fed the HF diet ($p<0.01$).

In the PF and RDC, acetic acid, propionic acid, butyric acid and iso-butyric acid were produced in significantly higher amounts in horses receiving the HF diet compared with those on the HS diet ($p<0.01$).

From the most distal intestinal compartment, the RE, higher levels of acetic acid, propionic acid and iso-butyric acid were found in samples from horses fed the HF diet ($p<0.01$). A significant diet*sex interaction was shown for iso-butyric acid content in the SF ($p=0.04$), RDC ($p<0.01$) and RE ($p=0.03$).

Table 15. Total VFAs (mg/100 ml) and individual VFAs expressed as a percentage (%) of total VFAs in the different intestinal compartments of the equine digestive tract according to the dietary treatment received (HS vs. HF). Data not normally distributed are expressed as medians (25^{th}–75^{th} percentiles); data normally distributed are expressed as means (SEM).

Intestinal compartments	VFAs	HS	HF	*p*-value		
				Diet	Sex	Diet*Sex
SI	Total VFAs	182.82 (130.20–235.04)	176.81 (142.89–238.49)	0.99	0.52	0.90
	Succinic	11.09 (3.00–14.29)	3.86 (0–16.87)	0.29	0.47	0.80
	Lactic	5.23 (3.60–7.83)	5.62 (2.96–26.27)	0.76	0.19	0.46
	Formic	0 (0–1.04)	0 (0–2.59)	0.92	0.05	0.92
	Acetic	55.37 (4.15)	51.32 (5.44)	0.63	0.54	0.65
	Propionic	0 (0–0)	0 (0–0)	0.74	0.26	0.74
	Iso-butyric	24.84 (11.05 – 32.57)	16.41 (11.63 – 24.66)	0.55	0.02*[a]	0.50
	Butyric	0 (0–0)	0 (0–0)	1.00	1.00	1.00
	Iso-valeric	0 (0–0)	0 (0–0.80)	0.38	0.38	0.38
	Valeric	0 (0–0)	0 (0–0.88)	0.59	0.64	0.75
CAE	Total VFAs	510.72 (261.05–771.56)	389.17 (290.76–462.55)	0.57	0.95	0.30
	Succinic	0 (0–0)	0 (0–0)	0.84	0.36	0.84
	Lactic	5.90 (2.43–6.84)	3.57 (2.79–6.65)	0.55	0.74	0.66
	Formic	0.50 (0.22–0.91)	0 (0–0.99)	0.10	0.01*[b]	0.97
	Acetic	20.19 (14.00)	30.16 (1.07)	<0.01*	0.88	0.53
	Propionic	4.19 (3.69–5.31)	9.05 (6.91–10.72)	<0.01*	0.32	0.79
	Iso-butyric	45.09 (43.05–52.16)	51.84 (47.31–56.48)	<0.01*	0.11	0.90
	Butyric	2.01 (1.66–2.18)	3.79 (2.85–4.23)	<0.01*	0.31	0.54
	Iso-valeric	0 (0–0)	0 (0–0)	0.38	0.38	0.38
	Valeric	19.26 (15.00–27.36)	0 (0–0)	<0.01*	0.64	0.64

SF	Total VFAs	838.11 (622.74–1054.03)	436.14 (381.38–489.42)	<0.01*	0.87	0.09
	Succinic	0 (0–1.21)	0.50 (0.31–0.95)	0.19	0.35	0.79
	Lactic	5.70 (0.10–6.66)	6.73 (4.88–12.33)	0.06	0.62	0.36
	Formic	0.32 (0.27–1.11)	0.65 (0–1.48)	0.74	0.58	0.47
	Acetic	17.71 (1.51)	33.42 (1.73)	<0.01*	0.29	0.08
	Propionic	3.80 (2.55–4.44)	8.67 (7.01–9.74)	<0.01*	0.40	0.63
	Iso-butyric	39.29 (37.77–42.78)	43.90 (36.91–49.68)	0.31	0.34	0.95
	Butyric	2.72 (2.54–3.26)	6.19 (5.35–7.29)	<0.01*	0.52	0.29
	Iso-valeric	0 (0–0.14)	0 (0–0)	0.51	0.37	0.69
	Valeric	30.74 (26.19–36.94)	0 (0–0)	<0.01*	0.06	0.06
PF	Total VFAs	897.97 (804.56–1121.135)	303.72 (235.53–342.82)	<0.01*	0.30	0.50
	Succinic	0 (0–0.68)	0.47 (0–1.42)	0.50	0.52	0.17
	Lactic	1.94 (0.23–3.92)	1.93 (0.90–2.44)	0.92	0.96	0.21
	Formic	0 (0–0.65)	0.23 (0–1.19)	0.51	0.68	0.25
	Acetic	13.93 (1.58)	36.61 (0.75)	<0.01*	0.27	0.49
	Propionic	2.48 (2.23–3.64)	9.60 (8.08–10.82)	<0.01*	0.22	0.75
	Iso-butyric	36.44 (32.23–42.31)	47.17 (43.81–48.63)	<0.01*	0.28	0.04*[c]
	Butyric	2.04 (1.31–2.30)	4.10 (2.54–5.45)	<0.01*	0.13	0.72
	Iso-valeric	0 (0–0.20)	0 (0–0.47)	0.88	0.94	0.99
	Valeric	43.34 (36.43–43.32)	0 (0–0)	<0.01*	0.33	0.33
RDC	Total VFAs	835.54 (672.89–1090.51)	284.62 (202.24–340.58)	<0.01*	0.58	0.41
	Succinic	0.10 (0–0.56)	0 (0–0.60)	0.46	0.10	0.88
	Lactic	0.25 (0–3.29)	3.03 (1.40–3.80)	0.07	0.58	0.60
	Formic	0 (0–0.19)	0 (0–0.21)	0.76	0.63	0.25
	Acetic	15.93 (1.86)	36.31 (2.29)	<0.01*	0.16	0.72
	Propionic	3.40 (2.73–3.84)	8.92 (6.84–9.46)	<0.01*	0.40	0.52

	Iso-butyric	39.10 (25.67–41.05)	46.62 (41.49–56.17)	<0.01*	0.14	<0.01*[d]
	Butyric	1.78 (1.44–4.16)	3.20 (2.35–4.16)	<0.01*	0.56	0.98
	Iso-valeric	0.18 (0–0.59)	0 (0–0)	0.06	0.13	0.43
	Valeric	39.25 (36.39–52.21)	0 (0–0)	<0.01*	0.33	0.33
RE	Total VFAs	605.76 (585.70–916.29)	195.39 (134.52–295.35)	<0.01*	0.21	0.83
	Succinic	0 (0–1.02)	0 (0–0)	0.08	0.58	0.58
	Lactic	3.04 (0.34–5.89)	3.32 (2.14–4.13)	0.61	0.08	0.28
	Formic	0 (0–0.23)	0 (0–1.06)	0.70	0.08	0.70
	Acetic	13.13 (1.37)	28.82 (1.69)	<0.01*	0.05	0.71
	Propionic	2.08 (1.93–3.72)	6.61 (5.89–8.80)	<0.01*	0.49	0.31
	Iso-butyric	37.12 (30.67–52.77)	62.16 (53.42–64.11)	<0.01*	0.29	0.03*[e]
	Butyric	1.45 (0.79–2.11)	2.00 (0.93–2.60)	0.92	0.17	0.95
	Iso-valeric	0 (0–0)	0 (0–0)	0.26	0.26	0.26
	Valeric	44.59 (19.98–51.79)	0 (0–0)	<0.01*	0.11	0.11

HS=high starch; HF=high fibre; SI=small intestine; CAE=apex of the caecum; SF=ventral diaphragmatic flexure of the colon; PF= pelvic flexure; RDC=right dorsal colon; RE=rectum; *statistical significance $p<0.05$.

*[a] males 30.50 (19.19–38.45); females 15.68 (7.97–22.90).

*[b] females 0.71 (0.12–1.33); males 0 (0–0.45).

*[c] males HF 47.99 (46.75–50.55)^{A}; females HF 46.44 (40.00–47.66)^{A}; females HS 40.78 (34.74–50.79)^{AB}; males HS 32.90 (28.32–36.35)^{B}. ^{A,B} $p<0.05$.

*[d] males HF 52.79 (47.17–60.11)^{A}; females HF 43.19 (38.83–55.10)^{A}; females HS 40.92 (35.16–48.41)^{A}; males HS 25.67 (18.70–31.67)^{B}. ^{A,B} $p<0.05$.

*[e] males HF 63.42 (62.30–64.41)^{A}; females HF 57.33 (50.34–60.02)^{A}; females HS 37.68 (36.17–66.13)^{AB}; males HS 30.67 (23.87–37.92)^{B}. ^{A,B} $p<0.05$.

4.5.5. Morphometric and histopathological findings

According to Table 16, no significant differences were recorded for all the evaluated morphometric indices in duodenum. In jejunum Vh/Cd was influenced by sex, being greater in males than in females (p=0.03) while in ileum the Cd was significantly affected by the interaction between sex and diet, being greater in males of HS group (p=0.03).

The histopathological findings are reported in Table 17. Significant differences were found in the duodenum and in the right dorsal colon according to the sex of the animals. In particular, female horses showed greater lymphoplasmacytic inflammation in the duodenum (p=0.02) and in the right dorsal colon (p=0.05) than male horses.

Moreover, the lymphoplasmacytic inflammation was significantly more severe in the jejunum (p=0.01) and in the pelvic flexure (p=0.05) of the horses fed the HS diet compared to the horses fed the HF diet. Instead, no statistical differences were recorded according to the other studied intestinal segments.

Table 16. Morphometric indices of duodenum (DU), Jejunum (JEJ) and Ileum (ILE) according to the two dietary treatments (HS vs. HF), sex and their interaction. Data are expressed as mean (±SD).

Intestinal segment	Morphometric indices	Diet		Sex		*p*-value		
		HS	HF	Female	Male	Diet	Sex	Diet*Sex
DU	Villus height (Vh)	0.39 (0.07)	0.36 (0.07)	0.38 (0.09)	0.37 (0.03)	0.32	0.70	0.71
	Crypt depth (Cd)	0.13 (0.02)	0.05 (0.01)	0.11 (0.19)	0.05 (0.01)	0.35	0.351	0.34
	Vh/Cd ratio	6.92 (1.30)	6.80 (1.43)	6.62 (1.39)	7.18 (1.26)	0.97	0.44	0.69
	Villus width	0.18 (0.53)	0.17 (0.02)	0.19 (0.05)	0.17 (0.02)	0.42	0.32	0.98
	Mucosa thickness	0.66 (0.11)	0.65 (0.08)	0.67 (0.11)	0.65 (0.06)	0.70	0.86	0.50
	Villus absorptive area	0.22 (0.06)	0.20 (0.06)	0.22 (0.07)	0.19 (0.03)	0.20	0.26	0.73
JEJ	Villus height	0.37 (0.03)	0.42 (0.09)	0.39 (0.09)	0.41 (0.05)	0.12	0.16	0.47
	Crypt depth	0.05 (0.10)	0.05 (0.007)	0.06 (0.01)	0.09 (0.11)	0.10	0.07	0.35
	Vh/Cd ratio	6.67 (2.95)	8.45 (2.10)	7.35^{A} (2.10)	7.96^{B} (3.35)	0.08	0.03*	0.99
	Villus width	0.19 (0.02)	0.20 (0.03)	0.19 (0.03)	0.19 (0.01)	0.99	0.93	0.35
	Villus absorptive area	0.22 (0.03)	0.26 (0.10)	0.24 (0.10)	0.24 (0.03)	0.18	0.19	0.85
ILE	Villus height	0.44 (0.04)	0.43 (0.07)	0.44 (0.06)	0.43 (0.05)	0.70	0.10	0.82
	Crypt depth	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.22	0.46	0.03
	Vh/Cd ratio	8.10 (1.11)	8.86 (2.27)	8.44 (2.12)	8.58 (1.41)	0.56	0.63	0.25
	Villus width	0.19 (0.03)	0.20 (0.02)	0.20 (0.02)	0.19 (0.03)	0.32	0.45	0.20
	Mucosa thickness	0.75 (0.11)	0.77 (0.16)	0.77 (0.16)	0.74 (0.10)	0.81	0.83	0.18
	Villus absorptive area	0.26 (0.06)	0.28 (0.06)	0.27 (0.05)	0.27 (0.07)	0.77	0.70	0.63

* Statistical significance $p<0.05$; HS=high starch; HF=high fibre

Table 17. Histopathological findings of duodenum (DU), Jejunum (JEJ), Ileum (ILE), apex of the caecum (CAE), sternal flexure (SF), pelvic flexure (PF), right dorsal colon (RDC) and rectum (RE) according to the dietary treatments (HS vs. HF), diet and their interaction. Data are expressed as mean (±SD).

Inflammation	Diet		Sex		*p*-value		
	HS [1]	HF [2]	Female	Male	Diet	Sex	Diet*Sex
DU	1.72 (0.79)	1.40 (0.57)	1.73[A] (0.65)	1.31[B] (0.70)	0.22	0.02*	0.52
JEJ	1.89 (0.55)	1.40 (0.66)	1.77 (0.75)	1.44 (0.42)	0.01*	0.14	0.35
ILE	3.22 (1.28)	2.50 (1.35)	3.18 (1.63)	2.36 (0.58)	0.09	0.15	0.35
CAE	4.89 (0.48)	4.60 (0.97)	4.91 (0.77)	4.50 (0.75)	0.40	0.28	0.85
SF	2.89 (0.99)	2.55 (0.50)	2.77 (0.93)	2.63 (0.52)	0.43	0.47	0.73
PF	3.22 (0.67)	2.80 (0.75)	3.18 (0.72)	2.75 (0.71)	0.05*	0.09	0.91
RDC	3.17 (1.06)	3.00 (1.07)	3.14[A] (0.95)	2.25[B] (0.96)	0.13	0.05*	0.72
RE	3.00 (1.03)	2.40 (0.77)	2.773 (1.15)	2.56 (0.56)	0.11	0.62	0.99

* Statistical significance $p<0.05$; HS=high starch; HF=high fibre

4.5.6. Microbiological contamination of mesenteric lymph nodes and liver samples

According to Raspa et al., 2021 [95], Table 18 shows the results on the Total mesophilic aerobic bacteria counts and Enterobacteriaceae counts. TMABc were found increased in HS than in HF for both mesenteric lymph nodes ($p=0.04$) and liver samples ($p=0.05$), indicating a different microbial contamination in those tissues according to the dietary treatment. No differences between HS and HF were found in mesenteric lymph nodes ($p=0.31$) and liver samples ($p=0.11$) for Enterobacteriaceae counts. Moreover, no samples were found to be contaminated by *Salmonella* spp. or *Escherichia Coli.*

Table 18**.** TMABc (Total mesophilic aerobic bacteria counts) and Enterobacteriaceae counts according to the two dietary treatments (HS vs. HF): median values (25^{th}–75^{th} percentiles) expressed as CFU/g. Adapted from Raspa et al., 2021 [95].

		HS [1]		HF [2]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
Mesenteric lymph nodes	TMABc	$36*10^2$ ($7*10^2$–$83.75*10^2$)	$4*10^2$ ($1.75*10^2$–$13.75*10^2$)	$2*10^2$ ($1*10^2$–$4*10^2$)	$2*10^2$ ($1.50*10^2$–$2.50*10^2$)	0.04*	0.34	0.09
	Enterobacteriaceae	55 (10–90)	5 (0–10)	10 (0–20)	0 (0–10)	0.19	0.21	0.42
Liver	TMABc	$11.50*10^2$ ($4*10^2$–$127*10^2$)	$38.25*10^2$ ($4.38*10^2$–$70.25*10^2$)	$1*10^2$ ($1*10^2$–$2*10^2$)	$1*10^2$ (0–$7*10^2$)	0.05*	0.28	0.95
	Enterobacteriaceae	20 (0–55)	25 (2.5–70)	0 (0–10)	0 (0–20)	0.11	0.69	0.85

* Statistical significance $p<0.05$; [1] High starch (n=9); [2] High fibre (n=10)

4.6. Feeding management: behaviour

Over the 96 h video-recordings, a total amount of 144 scans per horse were performed each day, providing a total of 10,368 scans sampled.

Differences in behavioural activities of horses according to the diet (HS vs. HF) are shown in Table 19. Playing behaviour was more frequently engaged in HS than HF (3.06% ± 0.10% vs. 1.92% ± 0.07%, $p<0.01$). Locomotion was observed more frequently in horses belonging to HS than HF (13.63% ± 0.61% *vs* 7.44% ± 0.77%, $p<0.01$). Instead, the feeding behaviour was more expressed by HF than HS (25.77% ± 0.38%, $p<0.01$) and it resulted as the main expressed behavioural activity engaged in by horses belonging to HF. On the contrary, the main expressed behavioural activity performed by horse in HS was represented by standing. Standing behaviour occupied the 30.29% ± 0.60% of time in HS and it was more frequent than HF (24.82% ± 0.57%, $p<0.01$). The HF group seemed also more engaged in snaking with the 0.08% (0.00%–0.23%) of time ($p<0.01$), whereas this behaviour was never recorded in HS. Horses belonging to HS were observed more frequently engaged in biting than HF (0.08% ± 0.02% vs. 0.02% ± 0.01%, $P=0.02$). Finally, stereotypic behaviour which included both oral and locomotor stereotypies were more frequently expressed in HS than HF (0.38% ± 0.04% vs. 0.07% ± 0.01%).

Table 19. Frequency of time (%) engaged in behavioural activities by horses belonging to HS (high starch group) and by horses belonging to HF (high fibre group). All data are expressed as mean ± SEM, except for snacking and kicking which are expressed as median (plus 25^{th}–75^{th} quantiles).

Behavioural activities	HS	HF	*p*-value
Self-grooming	0.83 ± 0.20	0.33 ± 0.15	0.06
Mutual grooming	1.52 ± 0.33	1.52 ± 0.35	1.00
Lying	22.65 ± 1.19	20.82 ± 0.56	0.18
Playing	3.06 ± 0.10	1.92 ± 0.07	<0.01*
Locomotion	13.63 ± 0.61	7.44 ± 0.77	<0.01*
Feeding	25.77 ± 0.38	40.21 ± 0.69	<0.01*
Drinking	1.68 ± 0.34	2.61 ± 0.32	0.06
Standing	30.29 ± 0.60	24.82 ± 0.57	<0.01*
Snaking	0.00 (0.00–0.00)	0.08 (0.00–0.23)	<0.01*
Kicking	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.15
Biting	0.08 ± 0.02	0.02 ± 0.01	0.02*
Sexual behaviour	0.09 ± 0.02	0.12 ± 0.04	0.47
Stereotypic behaviour	0.38 ± 0.04	0.07 ± 0.01	<0.01*

*Statistical significance $p<0.05$; HS=high starch; HF=high fibre

4.7. Feeding management: production performances

4.7.1. Growth performances

The data reported in Table 20 are adapted from Raspa et al., 2021 [95]. In particular, the Table shows the mean (SEM) initial bodyweight (iBW) of the horses of each group upon their arrival at the farm, the mean (SEM) slaughter bodyweight at end of the study (sBW) and the calculated average (SEM) daily bodyweight gain (ADG) for the two groups involved in the present study (HS and HF).
No differences in sBW according to diet, sex or their interaction were evident between the two groups of horses at the end of the trial. Moreover, ADG showed no differences in the two groups of horses according to dietary treatment, sex or their interaction.

Table 20. Mean (SEM) initial bodyweight (iBW), mean (SEM) slaughter bodyweight (sBW) at the end of the trial (129 days) and the calculated mean (SEM) daily bodyweight gain (ADG) for the two groups of horses (HS and HF). Adapted from Raspa et al., 2021 [95].

	HS [1]		HF [2]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
iBW	216.6 (4.02)	218.75 (5.44)	222 (2.07)	219 (2.08)	-	-	-
sBW	346.6 (2.42)	349 (4.38)	343.43 (0.92)	346.67 (1.76)	0.14	0.22	0.61
ADG	1.01 (0.03)	1.01 (0.03)	0.94 (0.02)	0.99 (0.02)	0.15	0.20	0.57

* Statistical significance $p<0.05$; [1] High starch group (n=9); [2] High fibre group (n=10);

4.7.2. Muscle characteristics and chemical composition of the *Longissimus thoracis et lumborum muscle*

Table 21 shows the mean values (SEM) of the muscle characteristics and the chemical composition of the *Longissimus thoracis et lumborum muscle* samples obtained from horses according to the two dietary treatments (HS vs. HF). The pH and the water holding capacity were lower in HS vs. HF according to the diet ($p=0.02$ and $p=0.04$, respectively). Moreover, the water holding capacity resulted to be affected by the sex of the animals ($p=0.03$) since *Longissimus thoracis et lumborum muscle* from females in HS showed lower water holding capacity than that of females in HF. Moreover, muscle colour in HS was characterised by increased lightness (*L*) ($p=0.01$) compared with muscle samples from HF. Regarding the chemical composition of the muscle, lower moisture content ($p=0.03$), increased protein content ($p=0.01$) and increased concentration of intramuscular fat (IMF) ($p=0.03$) was found in muscle samples from horses in HS compared with those from HF according to the diet. No differences were observed in ash concentration between the two groups.

Table 21. Characteristics and chemical composition of Longissimus thoracis et lumborum muscle (HS vs. HF). Data are expressed as mean (SEM). Adapted from Raspa et al., 2021 [95].

	HS [1]		HF [2]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
pH	6.68 (0.06)	6.70 (0.05)	6.49 (0.07)	6.54 (0.07)	0.02*	0.63	0.85
Water holding capacity (%)	80.27 (0.42)^{A}	81.27 (0.81)^{AB}	82.37 (0.32)^{B}	81.17 (0.08)^{AB}	0.04*	0.83	0.03*
Haematin (µg/g)	250.31 (17.88)	236.19 (38.99)	229.87 (2652)	259.5 (68.66)	0.97	0.83	0.55
L [3]	38.65 (0.58)	39.20 (1.39)	36.23 (0.57)	37.00 (0.28)	0.01*	0.44	0.90
a [4]	16.46 (0.60)	16.65 (0.46)	17.33 (0.22)	16.39 (0.39)	0.50	0.41	0.22
b [5]	-2.46 (0.48)	-1.50 (0.38)	-1.55 (0.18)	-1.04 (0.47)	0.09	0.07	0.56
L* [6]	36.86 (1.29)	37.70 (1.34)	35.98 (0.23)	37.46 (0.28)	0.57	0.24	0.74
a* [7]	15.96 (0.55)	16.75 (0.56)	16.71 (0.35)	16.26 (0.38)	0.71	0.73	0.23
b* [8]	-1.53 (0.34)	-1.01 (0.58)	0.44 (0.17)	-1.20 (0.33)	0.71	0.21	0.88
Moisture (%)	70.44 (0.20)	70.48 (0.51)	71.49 (0.31)	71.63 (0.88)	0.03*	0.84	0.90
Protein (% of DM [9])	75.86 (1.27)	75.34 (2.11)	79.37 (0.82)	80.23 (1.90)	0.01*	0.91	0.64
IMF [10] (% of DM)	11.8 (1.92)	13.08 (2.99)	8.31 (0.85)	7.08 (1.58)	0.03*	0.99	0.52
Ash (% of DM)	4.30 (0.32)	4.83 (0.52)	4.94 (0.29)	4.67 (0.52)	0.56	0.76	0.33

* Statistical significance $p<0.05$; ^{A,B} Means with different superscripts differ at $p<0.05$; [1] High starch (n=9);[2] High fibre (n=10); [3] Lightness on fresh samples; [4] Redness on fresh samples; [5] Yellowness on fresh samples; [6] Lightness after thawing; [7] Redness after thawing; [8] Yellowness after thawing; [9] Dry matter; [10] Intramuscular fat

4.7.3. Fatty acid profile of the *Longissimus thoracis et lumborum* muscle

The fatty acid profiles of muscle samples from horses reared using different feeding managements (HS vs. HF) are reported in Table 22. Muscle from horses fed with high amounts of fibre showed an increased concentration of C20:5 (p=0.03), PUFA (p=0.05) and n6 (p=0.04) than muscle from horses fed with high amounts of starch.

Table 22. Fatty acid profile (expressed as % of fatty acid methyl esters) of Longissimus thoracis et lumborum muscle samples (HS vs. HF). Data reported are expressed as mean (SEM). Adapted from Raspa et al., 2021 [95].

	HS [1]		HF [2]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
C10:0	0.06 (0.01)	0.05 (0.00)	0.05 (0.01)	0.05 (0.00)	0.25	0.22	0.97
C12:0	0.10 (0.01)	0.11 (0.00)	0.12 (0.01)	0.12 (0.02)	0.24	0.25	0.79
C14:0	2.01 (0.26)	2.30 (0.44)	2.25 (0.44)	1.95 (0.12)	0.70	0.87	0.84
C15:0	0.61 (0.17)	0.53 (0.05)	0.55 (0.06)	0.61 (0.18)	0.76	0.83	0.96
C16:0	28.11(0.69)	27.13 (0.61)	27.05 (0.74)	28.55 (1.40)	0.84	0.77	0.17
C16:1	4.81 (0.27)	4.97 (0.35)	5.00 (0.27)	5.37 (0.37)	0.38	0.42	0.74
C17:0	2.94 (0.48)	3.20 (0.63)	4.45 (1.04)	3.08 (1.87)	0.53	0.62	0.46
C18:0	7.04 (0.45)	6.58 0.41)	6.76 (0.56)	7.14 (0.63)	0.81	0.94	0.48
C18:1	30.42 (0.41)	30.74 (0.47)	29.42 (0.75)	28.85 (2.17)	0.15	0.90	0.65
C20:0	0.12 (0.00)	0.12 (0.01)	0.12 (0.01)	0.15 (0.00)	0.17	0.14	0.15
C18:2n–6	17.23 (0.88)	18.13 (0.77)	17.67 (0.74)	17.16 (1.76)	0.79	0.85	0.49
C18:3n–6	0.02 (0.00)	0.03 (0.00)	0.03 (0.00)	0.03 (0.01)	0.93	0.96	0.62
C18:3n–3	4.55 (0.11)	4.32 (0.24)	4.52 (0.25)	4.84 (0.23)	0.33	0.87	0.28
C20:4n–6	0.64 (0.08)	0.53 (0.03)	0.66 (0.16)	0.74 (0.11)	0.59	0.82	0.32
C20:5n–3	0.02 (0.00)	0.02 (0.00)	0.07 (0.04)	0.03 (0.01)	0.03*	0.46	0.67
C22:0	0.41 (0.01)	0.41 (0.02)	0.45 (0.02)	0.42 (0.02)	0.26	0.58	0.44
C22:6n–3	0.88 (0.07)	0.88 (0.09)	0.87 (0.07)	0.93 (0.55)	0.81	0.73	0.71
SFA[3]	41.47 (0.52)	40.44 (0.71)	41.80 (0.63)	42.06 (0.89)	0.19	0.60	0.38
UFA [4]	58.57 (0.52)	59.60 (0.70)	58.23 (0.63)	57.96 (0.89)	0.18	0.60	0.37
MUFA [5]	35.23 (0.50)	35.71 (0.52)	34.41 (0.87)	34.23 (2.35)	0.30	0.89	0.76
PUFA [6]	22.57 (0.31)	22.89 (0.36)	24.52 (0.68)	24.90 (2.51)	0.05*	0.71	0.98
n3	5.34 (0.17)	4.98 (0.31)	5.50 (0.32)	5.74 (0.28)	0.32	0.65	0.27
n6	17.03 (0.33)	17.91 (0.06)	19.02 (0.48)	19.16 (2.23)	0.04*	0.49	0.62
n6/n3	3.08 (0.12)	3.62 (0.20)	3.51 (0.18)	3.33 (0.23)	0.75	0.40	0.11
SFA/UFA	0.71 (0.02)	0.68 (0.02)	0.72 (0.02)	0.73 (0.02)	0.15	0.62	0.46
SFA/MUFA	1.18 (0.02)	1.13 (0.02)	1.22 (0.04)	1.24 (0.10)	0.14	0.79	0.50
SFA/PUFA	1.79 (0.08)	1.71 (0.10)	1.77 (0.08)	1.79 (0.12)	0.72	0.75	0.62
AI [7]	24.38 (0.81)	24.92 (1.01)	24.87 (0.92)	24.80 (1.90)	0.87	0.84	0.79
TI [8]	2.11 (0.10)	1.89 (0.06)	2.01 (0.11)	2.26 (0.18)	0.27	0.90	0.07

* Statistical significance $p<0.05$; [1] High starch group (n=9); [2] High fibre group (n=10); [3] SFA: saturated fatty acids; [4] UFA: unsaturated fatty acids; [5] MUFA: monounsaturated fatty acids; [6] PUFA: polyunsaturated fatty acids; [7] AI: atherogenic index; [8] TI: thrombogenic index

4.7.4. Antioxidant enzymes and oxidation end-products

Table 23 shows the results obtained from oxidative enzyme analyses. Muscular GPx and muscular SOD were higher in samples from HS compared with those from HF according to the dietary treatment (p=0.01 and p=0.03), whereas plasma CAT was lower in samples from HS compared with those from HF (p=0.05). Of the biochemical metabolites resulting from oxidation pathways (Table 24), higher concentrations of muscular TBARs were evident in samples from HF compared with samples from HS (p=0.01).

Table 23. Plasma, muscle and hepatic concentrations of glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). Adapted from Raspa et al., 2021 [95].

		HS [1]		HF [2]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
GPx	Plasma (μmol/mg) Median (25^{th} -75^{th})	0.07 (0.07–0.07)	0.09 (0.05–0.16)	0.08 (0.05–0.14)	0.12 (0.07–0.14)	0.40	0.36	0.98
	Muscle (U/mg) Median (25^{th} -75^{th})	0.14 (0.12–0.23)	0.25 (0.15–0.26)	0.12 (0.11–0.14)	0.13 (0.11–0.14)	0.01*	0.38	0.47
	Liver (μmol/mg) Mean (SEM)	0.26 (0.01)	0.22 (0.02)	0.22 (0.02)	0.24 (0.02)	0.70	0.77	0.11
CAT	Plasma (μmol/mg) Median (25^{th} -75^{th})	0.84 (0.63–0.88)	1.04 (0.69–1.31)	1.41 (0.83–1.47)	1.20 (1.01–7.15)	0.05*	0.25	0.99
	Muscle (U/mg) Median (25^{th} -75^{th})	5.03 (3.13–7.04)	3.34 (2.67–4.57)	2.96 (2.78–3.46)	4.03 (2.47–5.52)	0.50	0.60	0.23
	Liver (μmol/mg) Median (25^{th} -75^{th})	536.89 (517.84–539.79)	519.7 (513.51–537.14)	522.06 (517.88–523.21)	534.79 (516.15–538.61)	0.84	0.92	0.16
SOD	Plasma (μmol/mg) Median (25^{th} -75^{th})	15.38 (7.88–19.07)	7.80 (4.97–14.73)	6.55 (6.29–17.93)	7.49 (7.02–14.03)	0.71	0.389	0.44
	Muscle (U/mg) Median (25^{th} -75^{th})	17.60 (14.68–18.26)	16.69 (15.59–18.33)	16.47 (5.48–17.53)	6.56 (5.91–17.54)	0.03*	0.66	0.61
	Liver (μmol/mg) Mean (SEM)	115.86 (1.98)	112.69 (3.90)	111.36 (2.33)	114.82 (1.80)	0.68	0.96	0.26

* Statistical significance $p<0.05$; [1] High starch group (n=9); [2] High fibre group (n=10); [a] Expressed as oxidised NADPH content.

Table 24. Plasma and muscle concentrations of TBARs, hydroperoxides and carbonylated proteins (HS vs. HF). Adapted from Raspa et al., 2021 [95].

		HS [1]		HF [2]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
TBARs	Plasma (nmol MDA[a]/ml) Mean (SEM)	1.33 (0.12)	1.15 (0.12)	1.33 (0.05)	1.17 (0.13)	0.90	0.11	0.94
	Muscle (mg MDA[a] /kg) Mean (SEM)	0.26 (0.02)	0.36 (0.07)	0.50 (0.06)	0.47 (0.07)	0.01*	0.57	0.31
Hydroperoxides	Plasma (µmol/l) Mean (SEM)	5.25 (0.43)	5.40 (0.58)	5.29 (0.22)	5.73 (0.45)	0.66	0.48	0.72
	Muscle µmol/g Median (25^{th} -75^{th})	0.46 (0.42–0.54)	0.69 (0.48–0.88)	0.55 (0.45–0.59)	0.5 (0.42–0.66)	0.81	0.23	0.11
Carbonilated proteins	Plasma (µmol/ml) Mean (SEM)	98.85 (3.63)	101.10 (10.18)	94.43 (5.71)	90.67 (15.97)	0.41	0.93	0.73
	Muscle (nmol DNPH/mg) Mean (SEM)	1.25 (0.19)	1.43 (0.13)	1.24 (0.12)	1.15 (0.16)	0.39	0.78	0.41

* Statistical significance $p<0.05$; [1] High cereal grains group (n=9); [2] High fibre group (n=10); [a] Expressed as malonaldehyde (MDA) content; [b] Expressed as dinitrophenylhydrazine (DNPH) content

5. DISCUSSION

In order to discuss the findings of the present PhD project in a manner as clear as possible, the discussion will be divided following the same structure of the materials & methods and results sections. In particular, the results related to the first aim of the present PhD project are discussed in the section 5.1. and adapted from Raspa et al., 2020a [75] and Raspa et al., 2020b [71].

Instead the results obtained to answer the second aim of the present PhD project are discussed in the sections 5.2.

5.1. Welfare assessment

5.1.1. Welfare assessment: stocking density, feeding management and welfare indicators

Nowadays, farms breeding horses for meat primarily rear young horses and apply intensive farming systems in order to reduce the length of the fattening period and to obtain fast increases in bodyweight [5,7]. However, concerns about animal welfare related to overcrowding – i.e. reduced space allowance per animal or high stocking density within group pens – and intensive feeding regimes – i.e. diets characterised by high amounts of energy dense feedstuffs rich in hydrolysable carbohydrate as starch and simple sugars – have been raised over intensive farming systems [119]. In fact, several studies have underlined the negative effects of high stocking density on the welfare of some livestock species [42–44]. However, to the best of our knowledge, no research today has focused on horses farmed for meat production. Therefore, the first aim of the present PhD project was to provide information about the welfare status of horses farmed for meat purpose. Accordingly, the results obtained in section 4.2. identifies some problems regarding this kind of intensive breeding system and describe the effects of the stocking density and the feeding management on the selected welfare indicators in horses reared for meat production.

The selected welfare indicators were collected within a welfare assessment checklist that was specifically developed for this purpose (see Table 3). The assessment of animal welfare should be carried out by means a combination of resource-, management- and animal-based indicators to describe the various aspects of animal welfare [120,121]. In the present study, it was applied a welfare assessment checklist based on the AWIN structure – i.e. characterized by the four WQ® principles: good feeding, good housing, good health and appropriate behaviour. However, since the AWIN protocol is a tool which aims to describe the welfare of animals according to the evaluation of animal-based indicators, other resource- or management- based indicators were included. In fact, as reported by AWIN, the AWIN protocol was developed for adult horses (more than 5 years old) and may be difficult to apply to horses housed in group pens. As suggested by AWIN, the AWIN protocol needs to be redefined in light of up-to-date scientific research on horses kept in group pens and the specific breeding system being applied. As reported in the results section (section 4.2.), the horses in the farming system studied were young (mean ±SD 16 ± 8 months of age) and housed in groups.

In the present study, the stocking density of group pens was calculated as m^2 available per horse (m^2/horse). Two stocking density cut-off values were calculated and considered in the statistical analyses, calculated as the median (3.95 m^2/horse) and the 75th percentile (4.75 m^2/horse) values in order to evaluate whether any improvements in horse welfare could be observed with even a small increase in space allowance per horse. In particular, the use of two different stocking density cut-off values enabled us to show that an increase of just 0.80 m^2/horse (3.95 to 4.75m^2/horse) was able to have a significant effect on specific welfare indicators.

Stocking density and adequate space allowance is an aspect really important to consider when horses are housed in group, since it is strictly linked to the opportunity for movement [52]. The opportunity for movement is known to play an important role in equine welfare, having a positive effect on both physical and mental health [122].

Recommendations relating to the minimum space needed per horse housed in groups are provided by the AWIN protocol that takes into consideration the horse's height, as measured at the withers. In the present study, the height of the horses within the group pens ranged between 120 and 160 cm (see Table 2). According to AWIN, horses in this height range require at least 7 m^2/horse; but none of the pens at the farm provided this amount of space per horse.

Considering the welfare principle of good feeding, it was shown a significant influence of the space at the feed bunk (m/horse) at both cut-off values. The median feeding space per horse at the feed bunk was always less than 1 m/horse — the minimal distance recommended in the Code of Practice for the Care and Handling of Equines [51] — when the stocking density cut-off value was set to 3.95 m^2/horse. Adequate feeding space per horse at the feed bunk is important in order to mimic physiological feed intake behaviour and limit competition for resources [52,88]. Under natural conditions, horses live in herds and generally forage at the same time [85], preferring to maintain a distance of at least 2 m from each other [123]. In the HSD^{50th} condition of the present study, where the median space at the feed bunk was just 0.6 m/horse, the number of horses scored as "thin" according to the BCS scoring system was significantly higher than in the LSD^{50th} condition, where the feed bunk space was closer to 1 m/horse (0.95 m/horse). This difference was no longer present when the median feed bunk space of both groups exceeded 1 m/horse (i.e., when the 75th percentile cut-off was applied). It is interesting to notice that the number of animals exhibiting feeding behaviour was always greater in the high stocking density groups, independent of which cut-off value was used (HSD^{50th} or HSD^{75th}). This shows that both feed bunk space and stocking density can reciprocally influence feeding behavior.

For the welfare principle of good housing, stocking density was found to have a significant influence upon coat cleanliness. The number of animals rated as having a dirty coat was consistently higher in the high stocking density groups (HSD^{50th} and HSD^{75th}) compared with those housed at a lower stocking density (LSD^{50th} and LSD^{75th}). The welfare indicator bedding quantity was also judged as inadequate in both HSD^{50th} and HSD^{75th}. Indeed, when the lower cut-off value was set to 3.95 m^2/horse (i.e., the 50th

percentile), the frequency of pens in the HSD^{50th} group scored as containing an inadequate quantity of bedding was 83.3%. Whereas when the cut-off value was set to 4.75 m^2/horse (i.e., the 75th percentile), the frequency of pens scored as having an inadequate quantity of bedding was 78.2%. According to these results, when more animals are housed together, the frequency of inadequate quantities of bedding and the frequency of animals with dirty coats are higher. Indeed, the frequency of pens judged as having an inadequate level of bedding cleanliness exceeded 70% at all the stocking densities tested. This latter result may be a consequence of the high frequency of pens (>90%) containing abnormal faeces. The high prevalence of diarrhoea on the farm is probably related to the high level of starch in their diet. When the level of dietary starch exceeds the digestive capacity of a horse's small intestine, undigested starch may reach the hindgut where it undergoes rapid fermentation. The changes that may occur in the hindgut environment as a consequence of this starch, such as a decrease in luminal pH and marked changes in the microbial population, may lead to diarrhoea and an increased risk of colic [1,34].

Considered together, the results show that the amount and cleanliness of the pens' bedding were insufficient to provide an adequate level of environmental hygienic quality, which, in turn, influenced the cleanliness of the horses' coats. Moreover, we might hypothesise that horses housed at a higher density were more likely to consume the straw bedding to satisfy their natural need for foraging, especially at times when hay was not available [124], and this may have exacerbated the problem**.**

With regard to the welfare principle of good health, a number of welfare indicators were influenced by the stocking density. The number of horses presenting clinical signs of a cough was significantly higher in the HSD^{50th} group (<3.95 m^2/horse) compared with LSD^{50th} (≥3.95 m^2/horse). It is well known that increasing the stocking density increases the risk for transmission of respiratory diseases in stabled horses [125]. Thus, an increase in the per horse space allowance of a pen would be expected to decrease the occurrence of this indicator. Indeed, the statistical significance disappeared when the 75th percentile cut-off was applied.

No significant differences between groups were noted for either tail condition or mane condition when the stocking density cut-off value was set to 3.95 m^2/horse (50th percentile) since the scores of both groups revealed a high prevalence of mane and tail damage. However, differences were identified when the higher cut-off was applied, with horses allocated ≥4.75 m^2/horse (LSD^{75th}) less likely to incur mane or tail damage. A damaged tail might also be related to a major parasitic infestation that could cause excessive pruritus and lead to rubbing-induced injuries. It should be emphasized that no parasite management program was in force on this farm. Moreover, higher stocking densities may correlate with greater levels of contact made with the metal rail bars. It should also be noted that horizontal metal rail bars were in place at the feed bunks; thus, the crest of the neck was obliged to come into close contact with the metal rail bars during feed intake. Differences between groups were noted in the feeding behaviour of animals, independent of the cut-off value applied. However, the mane and tail

condition differences were only noted when the higher cut-off value was applied, indicating that when animals were kept at stocking densities lower than 4.75 m^2/horse (HSD^{75th}), a higher number of animals incurred tail and mane damage, also due to the constraint of the metal rail bars at the feed bunks.

In addition to providing key information about health-related parameters, the direct observation of the animals is fundamental for gathering data on horse behaviour. Within the welfare principle of appropriate behaviour, it was observed that a higher number of animals were feeding in the more densely housed groups for both cut-off values (HSD^{50th} and HSD^{75th}). However, feeding behaviour was spot sampled and may not indicate a long-term behaviour pattern. In contrast, the BCS constitutes a more direct indicator of feed intake over time [22] and reflects the consequences of feeding behaviour over the previous weeks [126]. What is important to underline is that when the stocking density cut-off was set to 3.95 m^2/horse, we identified a higher number of animals judged as thin in the high stocking density condition. This may mean that space allowance can also influence the time dedicated to feeding. A reduction in the feeding space would be a problem if it precludes easy access to feed, which may also increase competition for resources and thus influence the daily growth rate [88].

Of the other welfare indicators describing appropriate behaviour, resting in a standing position also seemed to be influenced by the stocking density. The results suggest that the number of horses resting in a standing position was significantly higher in the groups characterized by a higher stocking density (HSD^{50th} and HSD^{75th}), which could be a consequence of the lack of space and physical restriction [127]. Interestingly, the other behaviours included in the checklist (mutual grooming, resting in lying position, playing, sexual behaviour, aggressive behaviour, and stereotypic behaviour) were only detected at a very low frequency or absent altogether. However, the sampling method used may have influenced the results, as these behaviours may occur at much lower frequencies, meaning that the spot sampling method was not sensitive enough to detect their expression. The expression of certain behaviours could even be masked by the sampling method, as may be the case for stereotypic behaviour [90]. Other authors have speculated that the absence of certain behaviours might be a sign of a state of apathy [128]. Horses may be particularly sensitive to unfavorable environmental conditions, which could induce them to show apathy and become less reactive to environmental stimuli [129,130]. This condition could lead to the development of "depressive syndromes", as reported by Fureix and colleagues [131]. Studies on the behavioural repertoire of horses reared for meat production are needed to investigate this possibility. Accordingly, the effects of stocking density on behavioural activies of horses reared for meat production were more studied by means of video-recordings and the results obtained are discussed in the following section (section 5.1.2.).

It is also important to consider the feeding management system used in this kind of breeding farm. Unfortunately, it was not possible to calculate the exact forage intake/animal/day. Nonetheless, we estimated that animals received approximately 6 kg of hay per day. Since hay was only supplied twice a day (7 am and 6 pm), we could presume – but then was confirmed by the video-recordings that were

performed on those animals – that horses spent long periods of time fasting during the day and night. Moreover, horses were fed 8 kg/animal/day of a cereal-based commercial pelleted feed that was high in starch. It is well known that feeding horses with high amounts of starch can affect their welfare, leading to gastrointestinal and behavioural disorders [24]. Indeed, a number of equine studies state that starch consumption should be limited to not more than 2 g starch/kg bodyweight (BW) per meal [24,33,34]–equivalent to no more than 1 kg of starch/meal for a 500 kg horse or 1820 g/meal of the commercial cereal-based pelleted feed used in the present study. At this farm, the horses received 4 kg/animal/meal of the cereal-based commercial pelleted feed, corresponding to 2.2 kg of starch/animal/meal. Although it was not possible to measure the BW of the horses involved in the present study, according to the breeder, the animals belonging to the Italian heavy draft breeds and the French heavy draft breeds weighed approximately 500 and 550 kg, respectively. Therefore, we can speculate that the amount of starch fed to the animals was approximately twice the recommended safe level.

The main limitation of the present study is related to the fact that all the assessments were made in a single farm, even though it is one of the biggest meat horse breeding farms in Italy. Moreover, it was not possible to have a control group in which the minimum requirements considered by AWIN were satisfied. Even with these limitations, the present study represents the first scientific attempt to assess the welfare of horses reared for meat production at a farm level. The data obtained show the need to understand more about the welfare of those animals, stimulating further investigations to elucidate the minimum space allowance per horse in a group pen required to generate improvements in horse welfare. Measures are also needed to improve the feeding management regimes used, which should consider the nutritional requirements and welfare of the horses and not just production goals.

5.1.2. Welfare assessment: stocking density and behavioural activities

The minimal space requirements proposed by the TSchV [49] and by the AWIN protocol in the section focused on group housed horsed [16] are reported to be not based on scientific evidence [50]. Accordingly, the present study had the practical implication to understand the minimal space requirement needed to guarantee the expression of behavioural indicators of positive welfare. In fact, the reduction in the horse's behavioural repertoire and/or the change in time-budget can reflect a low or inadequate welfare status [53,128]. Therefore, to compare the behaviours expressed by horses in human-managed environment with those expressed by wild or domesticated pasture horses allow to understand animal welfare in the former [81]. Differences are particularly evident when we talk about horses since despite the process of domestication, they have maintained the species-specific behaviours of their wild ancestors [82].

According to the results described in the section 4.3. of the present PhD thesis, the daily time-budget performed by horses reared for meat production was mainly expressed by standing (30.56% ± 6.56%),

feeding (30.55% ± 3.59%), and lying (27.33% ± 2.05%). Locomotion was engaged in 4.07% ± 1.06% of the time. By comparing these results with the data available in the literature about young (2–3 years old) wild-living horses, some important differences were observed. Przewalski horses spend 46.4% of the day feeding, 33.87% of the day standing, 7.4% of the day in locomotion, and 5.3% of the day lying down [94]. Duncan, in 1980 [132], reported similar data in young Camargue horses, which spend at least 56.37% of the daily time-budget engaged in feeding behaviour, 19.41% in standing behaviour, 6.97% lying down, and 5.55% of their time in locomotion, with variations according to the seasons. Taking these two studies into account, we can say that young wild-living horses have an overall time-budget in which feeding is the main expressed behavioural activity, followed by standing, lying, and locomotion. On the contrary, the daily time-budget of the horses of the present study reared for meat production involved standing as the main expressed behavioural activity, followed by feeding, lying, and locomotion. It seems that the environmental constraints imposed by the breeding farm resulted in these horses lying down more and moving less compared with Przewalski and Camargue horses.

The strong reduction in the expression of feeding behaviour is in accordance with the studies conducted by Yarnell et al., 2015 [133], and Benhajali et al., 2008 [53]. In the study by Yarnell et al., 2015 [133], horses housed in groups in a paddock area poor in grass spent 34.89% ± 14.3% of the time expressing feeding behaviour. As suggested by the same authors, this result was the consequence of the limited availability of grass. Moreover, in the study by Benhajali et al., 2008 [53], mares densely housed in paddocks were found to engage in feeding behaviour for 25.83% ± 26.80% of their time. These authors correlated this result with the lack of foraging opportunity. According to these two studies, our results could be interpreted in the same way, since animals were fed just twice a day with approximately 6 kg of hay/animal/day.

The reduction in feeding behaviour could also be linked to the lack of adequate space at the feed bunk, as shown in studies on other livestock species [134]. To this regard, the Code of Practice for the Care and Handling of Equines [51] recommends guaranteeing at least 1 meter feeding space per horse under group-housing conditions and suggests having an extra feeding point available (i.e., one feeding point more than the number of horses). However, none of the pens involved in the present study respected this indication.

The time spent standing by horses reared for meat production—30.56% ± 6.56%—was comparable with those reported in Przewalski horses at 33.87% [94]. In particular, our results show that a reduction in stocking density correlates with a reduction in the expression of standing behaviour during the night hours ($r = -0.68$, $p = 0.049$).

The time-budget of our study relating to lying behaviour—27.33% ± 2.05%—is in stark contrast with the data shown for wild-living horses. Yarnell et al., 2015 [133], reported their horses to spend just 0.08% ± 0.1% of the time lying down; and the mares studied by Benhajali et al., 2008 [53], never exhibited lying behaviour. From our results, it seems that the smaller pen areas may encourage horses

to lie down more, also because locomotion behaviour was found to increase as space availability increased. The reduction in the expression and/or the absence of lying behaviour is widely recognised as a sign of reduced welfare in domestic species [135,136]. However, little is known about the normal lying behaviours of horses over the course of 24 h periods, or about what factors affect lying in horses [137]. Heleski et al. [54] suggested that an increase in lying behaviour in weanlings housed in stalls could be due to boredom and the lack of possibility to perform other behaviours. Boredom and physical restriction may also be the reason for the high frequency of lying behaviour in the horses of our study. Moreover, in the present study no correlation was found between stocking density and lying behaviour frequency. Indeed, the overall increase in space allowance per horse was probably too small to allow for any differences. In fact, no guidelines or regulations are presently available for the housing and management conditions of horses reared for meat production. As clarified before, the only official issued by the European Union in relation to horse welfare is the Animal Welfare Indicators (AWIN) assessment protocol for horses [16] which suggests at least 7 m^2/horse when horse's height measured at the withers ranges from 140 to 150 cm – as those involved in the present study.

As a consequence, the limitation of this present study was related to the fact that it was not possible to have a control group in which the minimum space requirement considered by AWIN was satisfied.

Interestingly, the reduction in the stocking density within the group pens positively correlated with an increase in behavioural activities such as locomotion, playing, and self-grooming. Thus, having more space available allowed the horses to move and play more; these results are in accordance with studies carried out on other domestic species (e.g., dairy calves [138] and growing pigs [139]).

Increased active locomotion (e.g., active walk, trot, and canter) has been identified in relation to inappropriate housing conditions [53,140]. However, in our study, the increase in space per animal was correlated with an increase in the expression of slow walking and explorative behaviour (sniffing the ground whilst walking; see Table 5).

Playing behaviour and self-grooming have been identified as potential positive welfare indicators in many species [141–143]. In particular, although growing evidence suggests that an increase in playing behaviour in adult domestic horses could be related to inappropriate living conditions [144], it seems that young horses only express playing behaviour under favourable breeding conditions [128]. Therefore, an increase in playing behaviour according to an increase in the space available could be considered as a positive welfare indicator in young horses.

Since grooming is reported to be an expression of horse welfare [145], the increase in self-grooming according to the increase in the group pen space allowance may be linked to improved welfare and could be proposed as a positive welfare indicator in this kind of breeding farm. However, the significance of self-grooming as a positive behaviour is less clear than that of mutual grooming. In fact, it seems that when horses are kept in a group, they engage more in mutual grooming [141]. However, it has

also been suggested that the performance of self-grooming could be a sign of increased welfare (being a rewarding behaviour), as proposed for mutual grooming [141].

All the other behavioural activities occupied less than 7.49% of the total daily time-budget. The particularly low frequency of stereotypic behaviour is interesting to note. It is well known that an increased frequency of stereotypic behaviour may correspond with an animal's attempt to cope with an inadequate environment [146]. However, as a result of the imposed management conditions – i.e., the high stocking densities, the feeding regime used, and the impossibility to perform free movement – standing was the main expressed daily behavioural activity. Fureix et al., 2012 [131], showed that horses living under unfavourable welfare conditions can show apathy and unresponsiveness to environmental stimuli. Although in the present work it was not possible to study body position, in order to identify the apathetic state, the poor expression of stereotypic behaviours may be linked to a depressive state in these animals. The occurrence of stereotypic behaviours represents one of the most recognised behavioural indicators of welfare impairments. It could be supposed that the unusually low presence of stereotypic behaviours in horses reared for meat production could similarly reflect a condition of poor welfare.

5.2. Feeding management: gut health, behaviour, production performances

5.2.1. Feeding management: gut health

The second aim of the present PhD project was to evaluate the effects of two different diets – one based on high amounts of starch (HS) vs. one base on high amounts of fibre (HF) – on gut health, behaviour and production performances in horses reared for meat production.

Gut health is a multidimensional concept [56]. Accordingly, several aspects should be investigated in order to properly described the gut health: the structure and the functioning of the gastrointestinal barrier, the gut environment in terms of its microbial profile, the volatile fatty acids (VFAs) and the particle size distribution [36,77], and the digestion and absorption of nutrients [57].

Therefore, studying the effects of diet composition on the gastrointestinal environment as well as on the gastrointestinal barrier is important in order to ascertain the role of the diet on the development of diseases [31].

Although diet composition likely affects the health of all the different intestinal compartments, most studies in the equine field have used rectal faecal samples for their analyses, being easy and non-invasive to collect, meaning that direct evidence on the differential effects of diet on the distinct intestinal compartments remains sparse [79].

In the present study the dry matter (DM) content and ash content of faecal samples obtained from the right dorsal colon of the horses fed high quantities of grains (HS) were both significantly higher compared with samples obtained from horses consuming the HF diet. What it is interesting is that the DM content of the right dorsal colon was higher in the HS group, an effect that was due to diet only,

and not sex. This lies in agreement with the findings of Lopes and colleagues [147], who reported that feeding large amounts of grains reduced water content in the digesta of the right dorsal colon compared with a hay-only diet. Moreover, the same authors found that high level of grain ingestion resulted in marked changes in the right dorsal colon content and in the faeces that were more homogenous, dehydrated, foamy, and dense in comparison with the hay-only diet. The effect on the DM content may be due to different factors. One is related to the fact that feeding meals composed of high amounts of cereal grains causes postprandial dehydration as a consequence of the absorption of water from the colon [23]. Secondly, it is related to the fact that low forage intake causes less water consumption and a lower water content in the colon since eating forage stimulates water consumption and the forage itself holds water within the gastrointestinal tract [23,147]. We were unable to measure water intake in the present study, but the data obtained may support the finding by Lopes and colleagues [147] that high VFAs production may lead to greater levels of sodium and water absorption by the colonic mucosa. In fact, we observed significantly higher levels of total VFAs in horses fed the HS diet in all hindgut compartments (i.e. the sternal flexure, pelvic flexure, right dorsal colon and rectum) compared with those fed the HF diet. This finding corroborates those of other studies [35,148] which show that horses fed diets with a high starch content contain high concentrations of total VFAs across all segments of the intestinal tract.

Acetate, propionate and butyrate are reported to be the primary VFAs produced by bacterial fermentation within the equine gastrointestinal tract. In particular, the percentage of VFAs in caecal or colonic fluid is reported to be approximately 74% acetate, 17% propionate, 6% butyrate and 3–4% other VFAs (isobutyrate, valerate and isovalerate) [23].

A high total VFAs content may also increase the risk of digestive disturbances, such as colic, osmotic diarrhea and laminitis [34]. Moreover, variations in individual VFA produced may also play a role in the pathogenesis of certain symptoms typically associated with a high starch diet. According to the literature, increasing the proportion of cereal grains promotes the production of propionate and lactate at the expense of acetate [36,149,150]. Indeed, our results show that the percentage of acetate over total VFAs was lower in the horses fed the HS diet in all the gut compartments studied compared with the values obtained in the HF group. Moreover, our data revealed the percentage of propionate to be lower in horses fed the HS diet compared with those receiving the HF diet. By contrast, the percentage of butyrate produced in the caecum, pelvic flexure and right dorsal colon was higher in HF compared to HS. Wambacq et al. [28] reported butyric acid to be an end-product of the microbial fermentation of fibre, and proposed that it may promote gut health by increasing the differentiation of colonocytes, and exert an anti-inflammatory effect and modulate oxidative stress.

Changes in the relative proportions of individual VFAs suggest the occurrence of changes in microbial populations according to the type of diet consumed and the intestinal pH [26], both of which should be investigated in further detail. In particular, our study revealed higher amounts of total VFAs in the

HS group that were related to a significant increase in valeric acid, whereas no traces of valeric acid were detected in the HF group. Valeric acid represented around 40% of the total VFAs produced in the hindgut of the HS group. The significance and implications of its presence needs to be investigated and is of particular interest, especially considering the fact that this VFA is produced from lactate by lactate-utilizing bacteria following the former's accumulation in the case of a HS diet, as suggested by Grimm et al. [77]. It is interesting to underline that, according to Nadeau et al. [151], valeric acid has a high lipid solubility and is able to penetrate the mucosa. The same authors also reported that this VFA is important in the pathogenesis of gastric ulcers. Even if in the present study the production of valeric acid was not investigated in the stomach of the horses fed according to the two dietary treatments (HS vs. HF), it is interesting to notice that the horses belonging to the HS group showed more severe lesions in the glandular mucosa of the stomach compared to horses in HF group. Moreover, a statistical tendency was found for the squamous region of the horses in HS compared to those in HF (see Table 12). This effect related to the high production of valeric acid may also be of significance in the hindgut where inflammation processes have often been associated with high starch diets [24,34]. In fact, in the present study, the lymphoplasmacytic inflammation resulted more severe in the jejunum and in the pelvic flexure of the horses fed the HS diet compared to the horses fed the HF diet and this difference was related to the dietary treatment. Moreover, the sex of the animals resulted to influence the lymphoplasmacytic inflammation in the duodenum and in the right dorsal colon. In particular, the lymphoplasmacytic inflammation was greater in females than in males. The effect of sex on gut inflammation has not been investigated in horses until now. However, Kim et al. [152] suggested that sex influences the microbiome composition both in human and animals, probably as a consequence of the effect of sexual hormones. The authors reported that changes in microbiome composition could predispose females to a greater susceptibility of suffering from dysbiosis and subsequently gut inflammation. This could also explain the differences recorded in the present studies for gut morphometry between males and females, since gut microbiome can indirectly influence also the gut morphometry [153].

In order to prevent gastrointestinal disorders related to the starch intake, it has been suggested to not feed horses more than 2 g/starch/kg BW/meal [34] - thus, to not more than 1 kg of starch/meal for a 500 kg horse. According to Raspa et al., 2021 [95] (see Table 20) the mean (SEM, standard error of the mean) slaughter BW for the female horses belonging to the HS group was 346.6 (2.42) and male horses was 349 (4.38). They received 4 kg/animal/day of the starch-rich complementary feed corresponding to 1.98 kg of starch/horse/meal. Therefore, the amounts of starch fed to the horses in HS was almost three time higher than the safe level. Surprisingly, no effect on Vh (villus height), Cd (crypt depth) nor Vh/Cd ratio were found in the intestinal histo-morphology of duodenum, jejunum and ileum. However, the more severe lymphoplasmacytic inflammation found in HS compared to HF could explain the higher Total mesophilic aerobic bacteria counts (TMABc) in the lymph nodes and liver

samples found in the HS compared to HF. In fact, inflammation process could be responsible for alteration in the permeability of the intestinal barrier which may lead to higher bacterial translocation [31]. Regarding the Enterobacteriaceae counts of the liver samples, although no statistically significant difference was detected between groups, it is interesting to note that whilst Enterobacteriaceae were detected in the liver samples from HS, the median content in HF was zero.

A multitude of factors may trigger the intestinal barrier dysfunctions that generate a leaky gut, including infectious diseases, drugs, exercise or heat stress [154]. However, in agreement with Stewart et al., 2017 [31], it is possible to hypothesise that the diet was one of the main factors contributing to the differences between the groups of the present study [95].

In the horses fed a grain-rich, and thus starch-rich, diet (HS), we identified a higher ash content than found in horses fed the fibre-rich diet (HF). This was surprising as the ash intake was similar in the two diets (as reported in Table 7, HS=901.8 g ash as fed and HF=904.4 g ash as fed). However, the higher ash content in the intestinal tract of horses on the HS diet could be the result of lower amounts of ash absorption in the intestine as a direct consequence of the high amounts of starch fed in the diet [155]. On the contrary, the higher percentage of organic matter (OM) found in the sternal flexure, the pelvic flexure, the right dorsal colon and the rectum in the HF group could be related to the high fibre intake which has been reported to reduce the digestibility of OM [156,157]. Moreover, a diet*sex interaction was found in relation to the percentage of OM and ash content in the sternal flexure. The effect of sex was only seen in one of the intestinal compartments analysed, and more research is required to understand the basis of this observation.

To the best of our knowledge, the present study is the first to investigate differences in particle size distribution across the different compartments of the equine intestinal tract according to the diet consumed (HS vs. HF). For faecal samples obtained from the caecum of horses in the HS group, the fraction of particles that washed through the finest sieve (<1 mm) constituted 69.50% of the digesta. This finding is particularly interesting if we consider that the CAE is one of the most common sites – together with the ileum and the large colon pelvic flexure (PF) – of gastrointestinal tract obstruction or faecal impaction [158–160]. Moreover, in the sternal flexure, the pelvic flexure, the right dorsal colon and the rectum, our results showed that the proportions of faecal particles retained by a 8 mm sieve and washed through the finest sieve (<1 mm) were higher in horses fed the HS diet compared with those fed the HF diet. The finest particles made up around 50% of the total in the HS group, and this result could explain a finding by Lopes et al. [147], who described the digesta and faeces from horses fed a high starch diet to be more homogenous and dense compared with those from horses fed a hay only diet. In the literature, the majority of studies evaluating faecal particle size relate their findings to the dental status of the horse [161–163]. However, none of the horses involved in our study were affected by dental issues, being young healthy animals, so we could conclude that the differences found were related to the differences in the diets (HS vs. HF). Thus, our results suggest that the particle size

is not only influenced by chewing and the condition of the dental board but also by the amounts of starch supplied in the diet. In fact, it is well known that high amounts of undigested starch are responsible for alterations or shifts in microbiome composition, which lead to a reduction in the activity of fibrolytic microorganisms [31,77,164] and, as a consequence, a reduction in the fermentation capacity of the fibre [164,165]. This aspect seems particularly important if we consider that the adequate digestion of fibre is believed to be crucial for reducing particle retention in the intestine, the occurrence of which increases the risk of large colon impaction [160].

5.2.2. Feeding management: behaviour

The results described in the section 4.6. show that the feeding management adopted (HS vs. HF) had important consequences on the behaviour and subsequently on the welfare of horses reared for meat production.

In particular, the main observed behavioural activity engaged in by horses belonging to HF was feeding which was expressed for the 40.21% ± 0.69% of time; on the contrary in HS the feeding behaviour was observed only for the 25.77% ± 0.38% of time. The frequency of time spent feeding observed in HF was in quite alignment with the data available in the literature about young (2–3 years old) wild-living horses. In particular, Boyd et al., 1988 [94] showed that Przewalski horses spend 46.4% of the day on feeding behaviour. The increase of the time spent feeding in HF can be of course explained by the availability of hay and it could also clarify the reason why horses in HF were significantly less engaged in standing behaviour than HS (24.82% ± 0.57% vs. 30.29% ± 0.60%, respectively) and in locomotion (7.44% ± 0.77% vs. 13.63% ± 0.61% respectively). These findings are in agreement with Benhajali et al., 2009 [166] who studied the effects of increasing foraging opportunities on the behaviour of housed group mares. The authors correlated the increased foraging opportunity with the reduction of the expression of rest standing. Less time spent in alert standing and locomotion may be a sign of a lower welfare status in horses [166]. The common perception that excess energy from concentrate feeds causes "fizzy" or unwanted excitable behavior can be described by higher level of locomotion that represents a sign of agitation [72,167] and this may be the consequence of the high cereal-based diet which caused a high glycaemic response, resulting in increased reactivity behaviours [70]. It is interesting to discuss this higher level of locomotion as one of the factor that influence the production performances of those animals. In fact, the effects of these two feeding managements (HS vs. HF) on production performances of horses were also investigated and published in Raspa et al., 2021 [95]. Interestingly, it was found that the high amounts of cereal grains in the diet did not result with any difference in daily bodyweight gain (see Table 20). Therefore, economic repercussions need to be taken into account.

Moreover, as stated before in the section related to gut health, a diet based on high amounts of starch causes digestive discomfort caused by the overflow of undigested starch in the hindgut where it is rapidly fermented causing important changes in the gastrointestinal environment [24]. This condition may explain the higher incidence of stereotypic behaviour in HS compared with HF (0.38% ± 0.04 % vs 0.07% ± 0.01%, respectively).

Playing behaviour was found more expressed by HS than HF (3.06% ± 0.10% vs. 1.92% ± 0.07%, respectively). It is reported that growing horses express playing behaviour only under favourable breeding conditions and, accordingly, its expression can be considered as a positive welfare indicator [71]. However, it is also reported that play could be related to immediate short-term positive emotions [128]. Therefore, it appears difficult to explain the reason why HS showed this increase in playing behaviour compared with HF. Hausberger et al., 2012 [168] found out a relationship between adult play and altered welfare. Their study shows that adult horse play is not, as currently thought, a reliable welfare indicator. Future research should be carried out to discriminate different playing categories as described by Mcdonnel and Poulin, 2002 [169].

Biting was found more observed in HS than HF (0.08% ± 0.02% vs. 0.02% ± 0.01%, respectively). This behaviour is commonly associated with aggressive behaviour according to competitive situations – e.g. during foraging – [170]. Accordingly, the higher incidence of biting in HS may be related to the reduced availability of hay. Instead, snaking (herding with the head and neck extended and ears held back) was observed only in HF than HS. In feral herds, the approach of a foreign stallion evokes the snaking response in the harem stallion [171]. It was noted that snaking gestures from horses belonging to HF were performed with higher incidence towards the horses belonging to HS. Indeed, the two group pens were located side by side and each of which was enclosed by horizontal metal rail bars. Accordingly, the observation of snaking in HF can be considered as a sexual rather than an aggressive behaviour [172] with the aim to herding mares away from HS.

5.2.3. Feeding management: production performances

The results discussed in the present section are adapted by Raspa et al., 2021 [95].

What it is need to clarify is that the study was carried out under field conditions without any possibility of choosing the horses involved in the trial or to change the breeder's management choices for the HS. As a consequence, it was not possible to establish isoenergetic or isoproteic diets for the two experimental groups as it is possible to notice in Table 6.

In the present study, horses belonging to HS and HF were evaluated for several aspects related to production performances. In particular, selected traits between groups were explored, focusing on the muscle characteristics and chemical composition of the *Longissimus thoracis et lumborum* muscle. Muscle from female horses in HF showed a higher water holding capacity; and a higher moisture content and a lower pH were identified according to the dietary treatment. In both groups, muscle pH was

found to be higher than the values reported in other studies. For example, Gill, 2005 [2] reported the pH of horse muscle to be generally below 6. Similarly, Seong et al., 2017 [173] reported pH values around 5.75, with a significant increase in pH the longer samples had been stored (frozen). The low pH values reported in those studies are likely related to the fact that during the development of rigor mortis, muscle glycogen is converted to lactic acid [174]. After slaughter, glycolysis continues in tissues until the glycogen substrate is depleted, resulting in the accumulation of acidic glycolytic end-products and a drop in pH [175]. Our results suggest the existence of differences in the biochemical pathways (e.g. the glycolytic rate) underway in the muscle between groups. The high pH values detected in the present study could be due to different levels of muscle glycogen compared to the studies previously cited. Unfortunately, it was not possible to measure the muscular glycogen in this present study.

The values of water holding capacity recorded in this study were in agreement with the data reported in the literature on horse meat [6,8]. The significantly higher mean value found in the HF samples vs. those from HS could be due to the lower fat deposition between muscle fibres, the higher protein content and the higher moisture content [7]. A previous study found that increasing the requirements up to 200% in Italian Heavy Draft horses (IHDH) did not affect intramuscular fat content or the water holding capacity of muscle [104,106], but in those studies a different breed (IHDH) was studied compared the breed used in our study (Bardigiano). Moreover, the present study revealed a significant effect of feeding managment on both these muscle features. It is likely that the difference in results is due to the different characteristics of the feeding trials, which here focussed on different starch to fibre ratios. In addition, the animals fed HF were fed less protein and less fat and even the mineral composition was also different.

Even if the diets were not isoenergetic and isoproteic, some considerations should be taken into account. Interestingly, no statistical significance between groups was found in slaughter BW and ADG (see Table 20). According to the calculation of the net energy provided to the horses per day, the high starch diet supplied 42.3 MJ more than that provided by the diet characterised by high amounts of fibre. According to the French Institute National de la Recherche Agronomique (INRA), a daily body weight gain of 1 kg/day for a horse weighing 350 kg is possible if the animal is supplied with 14 MJ plus its maintenance requirement (46.1 MJ) [176]. These findings indicated that the extra energy level supplied with the high cereal grain diet did not result in a significantly higher daily body weight gain compared with that achieved in the horses of HF. This finding is surprising since horses in HS were fed more energy than horses in HF. Anyways, the HS diet overcomes the starch digestibility of 2 g of starch/kg BW as suggested by some authors [24,33]. Not all the estimated energy of the HS diet was used because the starch level in the diet exceeded the digestive capacity of the horse's intestine [24]. Moreover, an additional point that we should consider is that a HS diet can causes high glycaemic response, resulting in increased reactivity behaviors [70,72]. Horses in HS spent more energy in locomotion/reactivity behaviors than horses in HF as resulted by the evaluation of the video-recordings

performed on the two groups of horses (see Table 19). In conclusion the extra energy supplied with the HS diet is counterproductive, both from economic and welfare points of view.

Regarding colorimetric patterns, the fresh muscle samples from the HS group showed higher lightness values compared with those from HF, whereas these differences did not exist after thawing. Lightness in muscle is related both to the amount of intramuscular fat and to the water content on the cut surface [177]. Colour changes in meat from foals are affected by slaughtering age and post-thawing time [105]. The different IMF values could explain the tendency towards higher lightness values in muscle from HS compared with that from HF, both in fresh and in thawed meat. The significant differences in lightness in fresh muscle could be due to the different water holding capacities, whereas, after thawing and post-thawing water losses, the differences in lightness were not statistically significant. Moreover, muscle colour can also be affected by the fatty acid composition of IMF [5]; indeed, differences in the fatty acid profiles of the two groups were also revealed here.

The diet is one of the main factors influencing the concentration of IMF in horse muscle [5,178], and diet can influence the fatty acid profile of IMF [7]. In fact, several studies have recently underlined that horse breed, slaughter weight and management practices, including feeding management, affect the fatty acid composition of horses [9,10,179]. However, to the best of our knowledge, no studies have quantified the effects of a feeding management based on high amounts of fibre on the fatty acid composition of *Longissimus thoracis et lumborum* muscle of horses. Here, we found that the PUFA concentration was higher in muscle from HF compared with that from HS. In particular, this result was related to the higher concentration of n6 PUFAs and n3 eicosapentaenoic acid (EPA, C20:5n–3). These differences likely reflect differences between the two diets supplied. Among raw ingredients of the fibrous pelleted feed oilseeds (flaxseeds and dehulled sunflower seeds) was included at dose of 45 g/day during the final 72 days of the fattening period. Regarding the HS diet, the fat component was essentially supplied by the maize as a main ingredient. However, the total quantity of fat provided by the two diets was similar (see Table 7; HS=285.40 g, HF= 192.70 g; fat contribution to total energy content: HS=8.39%, HF=10.14%). Interestingly, although HS presented a higher IMF concentration, the HF was characterised by a better fatty acid profile, and this result could provide an important incentive to change the feeding practices of horses reared for meat production [180].

It has been shown that a higher IMF content results in lower moisture content [181,182]. Our data align with the literature since HS displayed a higher IMF content alongside with lower moisture. The mean moisture content was 70.5% and 71.5% for HS and HF muscles samples, respectively, in accordance with previous studies conducted on 11-24 months horses [7,8,179].

Horse muscle is characterised by a high protein content, which varies according to a number of factors, such as sex, muscle type and production system employed [5]. The French system [176] reports that for a daily growth of 1 kg BW, the total dietary protein requirements should be 733 g MADC/day for a horse weighing 350 kg (where MADC - Matières Azotèes Digestibles Cheval (MADC) - expresses horse

digestible crude protein, which represents the estimated measure of the quality of the absorbed amino acids provided by a diet). According to this, horses in the HF (with a mean sBW of 344.40 kg) would have needed to consume 692 g MADC/day for an average daily BW gain of 0.96 kg. In this study, the HF diet provided 723 g MADC/day. On the other hand, horses in the HGC (with a sBW of 347.8 kg) would have needed to consume 735 g MADC/day for a daily BW gain of 1.01 kg. However, the horses in HS were actually supplied with 1178 g MADC/day.

It is important to note that not only should the protein content of a feed meet the total MADC requirements, but also provide proteins of high biological value. In particular, in horse diets, lysine is the main limiting amino acid, especially if diets are cereal grain-based [183]. In fact, in our study, the horses in HS received an estimated 48 g of lysine in the diet. On the contrary, the high fibre group was supplied with 76.50 g of lysine. Therefore, these differences could have affected the development of the muscle.

Regarding oxidative status, the higher concentration of PUFAs in muscle samples from HF compared with that found in HS could explain the higher muscular concentration of TBARs in the HF. In fact, the different oxidative stability of IMF is reported to be related to the saturation index of fatty acids [184]. On the contrary, muscular GPx and muscular SOD were higher in HS than in HF. Although higher oxidative stress is related to lower GPx and SOD levels, the higher levels in HS compared with in HF remains unexplained. In particular, GPx activities are related to selenium intake, and a low selenium intake is related to low GPx activities and vice versa [185]. As shown in Table 7, the horses in HS received only 400 mg of Vitamin E and 0.48 mg of selenium per day, whereas those in HF were supplied with 1105 mg of Vitamin E and 1.72 mg of selenium. Selenium and Vitamin E are dietary antioxidants which synergistically support endogenous antioxidant systems to reduce reactive oxygen species damages. Limited data is reported from experimental feeding trials on effective nutritional supplementation in Vitamin E in horse meat. However, taking into account scientific studies carried out on other species [186,187], the α-tocopherol levels – natural isoform of the fat-soluble vitamin E group – in tissues and plasma were significantly influenced by the level of dietary supplementation, leading to higher stability of meat lipids. Moreover, Cappai et al., 2020 [188][188,189] recommended to monitor the Vitamin E intake in the context of adequate feeding practices for health and welfare assessment. In particular, since α-tocopherol is synthesized and stored chiefly in the green plants, the same authors suggested that a higher dietary intake of Vitamin E is important in stabled horses when they are fed on hay.

Finally, the higher plasma levels of CAT in the horses belonging to HF suggest that the animals tended to be protected from oxidative damage, as this enzyme is involved in one of the most rapid and effective systems for reducing oxygen free radicals [190]. A high fibre source in the diet can effectively promote antioxidant defence by enhancing the free radical-scavenging ability of the plasma and other relevant

organs [191]. However, no studies have been carried out to date on the antioxidative effects of dietary fibre intake and different fibre components on horse tissue.

6. CONCLUSIONS

The present PhD project applied an integrated approach to the evaluation of the welfare and management in the equine meat farm. In particular, several aspects – welfare indicators, gut health, behaviour and production performances – were taken into account according to two main aims.

The first aim of the present PhD project was to obtain insight into the housing and management welfare conditions of horses reared for meat production and to evaluate whether the selected welfare indicators and behavioural activities were influenced by the main causes of concern that regard intensive breeding farms: stocking density and feeding management. The results obtained revealed that stocking densities and feeding management influenced welfare indicators of horses reared in group pens for meat production and thus constitute key concerns. The results suggest that horse welfare is negatively affected by high stocking densities and the use of an intensive feeding management strategy. According to the results obtained, when the horses had more than 4.75 m^2/horse, many parameters were influenced (i.e., improvement of coat cleanliness, improvement of bedding quantity, improvement of the mane and the tail condition, less resting in a standing position and less feeding related to the greater space available at the feed bunk). Moreover, horses were fed rations rich in starch, which was probably responsible for the high incidence of diarrhoea and, consequently, the poor state of bedding cleanliness. Therefore, a further increment of space and changes in feeding management resulted necessary to improve the welfare status of horses reared for meat purpose.
The role of the stocking density was further studied investigating the effects of different stocking densities on the behavioural activities of the horses reared for meat purpose and subsequently on their welfare. Although the horses reared for meat production expressed an unusual time-budget, since, compared with wild-living horses, significantly more time was spent lying down and less time was dedicated to feeding and locomotion activities; the reduction in stocking density and as a consequence a space allowance of 6 m^2/horse had a positive impact on the expression of some behaviours – locomotion, playing, and self-grooming – which could be proposed as indicators of positive welfare in young horses kept in group pens.

The second aim of the present PhD project was to evaluate the effects of two feeding managements (on based on high amounts of starch vs. one based on high amounts of fibre) on gut health, behaviour and production performances in horses reared for meat production.
Regarding the gut health, the HS diet was found to have a profound effect on the horse's gut environment in terms of dry matter (DM), volatile fatty acids (VFAs) production and particle sizes as

well as on the horse's gastrointestinal barrier in terms of severity of gastric mucosa lesions, gut histo-morphometry and intestinal permeability.

A higher DM content in the right dorsal colon, a higher ash content and higher production of VFAs in all the analysed hindgut compartments were found in the horses fed the HS diet compared with horses fed the HF diet. Not only were total VFAs higher in the HS group, but differences in the VFA composition was also noted. In particular, the valeric acid was increased in horses receiving the HS diet, and this should be explored in more depth since this VFA has already been implicated in causing alterations to the gastric mucosa. In fact, the results obtained demonstrated that the HS diet was associated with the presence of more severe mucosa gastric lesions in the glandular region of the stomach and a higher lymphoplasmacytic inflammation in the jejunum and pelvic flexure; instead no differences were found regarding the histo-morphometry of duodenum, jejunum and ileum compared to the HF diet. Moreover, the results obtained supported the notion that feeding horses high amounts of starch can lead to a condition of increased intestinal permeability. In summary, the results of this study confirm that the diet composition, and thus feeding management practices, are able to influence the gut environment and its functioning.

Regarding the behavioural activities of horses reared for meat production according to the two dietary treatments (HS vs. HF), the present PhD project showed that the behavioural changes by feeding horses with a HF diet indicated increased welfare, according to the increased expression of the feeding behaviour and the reduced frequencies of standing and locomotion. Moreover, the HF feeding management resulted in a lower expression of stereotypic behaviour and biting. In summary, the change in feeding management from a HS diet to a HF diet in horses reared for meat production led to advantage on the horse's welfare since horses fed the HF diet showed less aggressive and stereotypic behaviours as well as on the economic point view since horses fed the HF diet were less engaged in by locomotion – so, spending less energy – and more occupied in feeding behaviour.

Accordingly, regarding to the production performances, the HS diet resulted wasteful from an economic stance since it did not result with any difference in daily bodyweight gain or with any positive effect on muscle characteristics. In fact, horses in HS showed increased muscle pH, lighter muscle colour, lower muscular protein content increased intramuscular fat concentrations but lower concentration of muscle polyunsaturated fatty acids (PUFAs) compared to the horses in HF. Moreover, the PhD study showed that diet influenced the concentrations of glutathione peroxidase, catalase and superoxide dismutase; although plasma, muscle and liver were characterised by distinct differences. Interestingly, the higher plasmatic catalase found in horses belonging to HF suggest that the animals were more protected by oxidative damages.

7. REFERENCES

1. Davidson, N.; Harris, P. Nutrition and Welfare. In *The welfare of horses*; Springer, Dordrecht,

2007; pp. 45–76.
2. Gill, C.O. Safety and storage stability of horse meat for human consumption. *Meat Sci.* 2005, *71*, 506–513.
3. Belaunzaran, X.; Bessa, R.J.B.; Lavín, P.; Mantecón, A.R.; Kramer, J.K.G.; Aldai, N. Horse-meat for human consumption - Current research and future opportunities. *Meat Sci.* 2015, *108*, 74–81.
4. FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QL.
5. Lorenzo, J.M.; Sarriés, M.V.; Tateo, A.; Polidori, P.; Franco, D.; Lanza, M. Carcass characteristics, meat quality and nutritional value of horsemeat: A review. *Meat Sci.* 2014, *96*, 1478–1488.
6. De Palo, P.; Maggiolino, A.; Centoducati, P.; Tateo, A. Slaughtering age effect on carcass traits and meat quality of italian heavy draught horse foals. *Asian-Australasian J. Anim. Sci.* 2013, *26*, 1637–1643.
7. Tateo, A.; De Palo, P.; Ceci, E.; Centoducati, P. Physicochemical properties of meat of Italian Heavy Draft horses slaughtered at the age of eleven months. *J. Anim. Sci.* 2008, *86*, 1205–1214.
8. Sarriés, M. V; Beriain, M.J. Carcass characteristics and meat quality of male and female foals. *Meat Sci.* 2005, *70*, 141–152.
9. Sarriés, M. V; Murray, B.E.; Troy, D.; Beriain, M.J. Intramuscular and subcutaneous lipid fatty acid profile composition in male and female foals. *Meat Sci.* 2006, *72*, 475–485.
10. Lorenzo, J.M.; Fuciños, C.; Purriños, L.; Franco, D. Intramuscular fatty acid composition of "Galician Mountain" foals breed Effect of sex, slaughtered age and livestock production system. *Meat Sci.* 2010, *86*, 825–831.
11. Broom, D.M. Animal Welfare in the European Union-Directorate General for Internal Policies. Policy Department C: Citizens' Rights and Constitutional Affairs Petitions. European Parliament Policy Department, Citizen's Rights and Constitutional Affairs, Study for the PETI Committee: Brussels, Belgium. Available online: https://op.europa.eu/en/publication-detail/-/publication/74df7b49-ffe7-11e6-8a35-01aa75ed71a1/ language-en.
12. Mason, G.; Mendl, M. Why is there no simple way of measuring animal welfare? *Anim. Welf.* 1993, *2*, 301–319.
13. Bøtner, A.; Broom, D.; Doherr, M.G.; Domingo, M.; Hartung, J.; Keeling, L.; Koenen, F.; More, S.; Morton, D.; Oltenacu, P.; et al. EFSA Panel on Animal Health and Welfare (AHAW). *EFSA J.* 2012, *10*.
14. Johnsen, P.F.; Johannesson, T.; Sandøe, P. Assessment of Farm Animal Welfare at Herd Level: Many Goals, Many Methods. *Acta Agric. Scand. A Anim. Sci.* 2001, *51*, 26–33.
15. Bertocchi, L.; Fusi, F.; Angelucci, A.; Bolzoni, L.; Pongolini, S.; Strano, R.M.; Ginestreti, J.; Riuzzi, G.; Moroni, P.; Lorenzi, V. Characterization of hazards, welfare promoters and animal-based measures for the welfare assessment of dairy cows: Elicitation of expert opinion. *Prev. Vet. Med.*

2018, *150*, 8–18.

16. AWIN European Union. AWIN Welfare Assessment Protocol for Horses. Available online: https://air.unimi.it/retrieve/handle/2434/269097/384836/AWINProtocolHorses.pdf.
17. Awin European Union. Awin Welfare Assessment Protocol for Donkeys. Available online: https://air.unimi. it/retrieve/handle/2434/269100/384805/AWINProtocolDonkeys.pdf.
18. Botreau, R.; Veissier, I.; Pern, P. Overall assessment of animal welfare: Strategy adopted in Welfare Quality®. *Anim. Welf.* 2009, *18*, 363–370.
19. Brambell, F.W.R. *Report of the Technical Committee to Equire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems*, 1st ed.; AW& Co Ltd.: Hemel Hempstead, UK, 1965.
20. Raspa, F.; Cavallarin, L.; McLean, A.K.; Bergero, D.; Valle, E.; Raspa, F.; Cavallarin, L.; McLean, A.K.; Bergero, D.; Valle, E. A Review of the Appropriate Nutrition Welfare Criteria of Dairy Donkeys: Nutritional Requirements, Farm Management Requirements and Animal-Based Indicators. *Animals* 2019, *9*, 315.
21. Henneke, D.R.; Potter, G.D.; Kreider, J.L.; Yeates, B.F. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet. J.* 1983, *15*, 371–372.
22. Becvarova, I.; Pleasant, R.S.; Thatcher, C.D. Clinical Assessment of Nutritional Status and Feeding Programs in Horses. *Vet. Clin. North Am. - Equine Pract.* 2009, *25*, 1–21.
23. Merritt, A.M.; Julliand, V. Gastrointestinal physiology. In *Equine Applied and Clinical Nutrition*; E-Book: Health, Welfare and Performance; Elsevier Ltd.: Amsterdam, The Netherlands, 2013; pp. 3–32.
24. Durham, A.E. The Role of Nutrition in Colic. *Vet. Clin. NA Equine Pract.* 2009, *25*, 67–78.
25. Eckersall, P.D.; Aitchison, T.; Colquhoun, K.M. Equine whole saliva: Variability of some major constituents. *Equine Vet. J.* 1985, *17*, 391–393.
26. Vondran, S.; Venner, M.; Vervuert, I. Effects of two alfalfa preparations with different particle sizes on the gastric mucosa in weanlings: alfalfa chaff versus alfalfa pellets. *BMC Vet. Res.* 2016, *12*, 1–8.
27. Andrews, F.M.; Larson, C.; Harris, P. Nutritional management of gastric ulceration. *Equine Vet. Educ.* 2017, *29*, 45–55.
28. Merritt, A.M. Normal equine gastroduodenal secretion and motility. *Equine Vet. J. Suppl.* 1999, *29*, 7–13.
29. Luthersson, N.; Hou Nielsen, K.; Harris, P.; Parkin, T.D.H. Risk factors associated with equine gastric ulceration syndrome (EGUS) in 201 horses in Denmark. *Equine Vet. J.* 2009, *41*, 625–630.
30. Sykes, B.W.; Hewetson, M.; Hepburn, R.J.; Luthersson, N.; Tamzali, Y. European College of Equine Internal Medicine Consensus Statement-Equine Gastric Ulcer Syndrome in Adult Horses.

J. Vet. Intern. Med. 2015, *29*, 1288–1299.

31. Stewart, A.S.; Pratt-Phillips, S.; Gonzalez, L.M. Alterations in Intestinal Permeability: The Role of the "Leaky Gut" in Health and Disease. *J. Equine Vet. Sci.* 2017, *52*, 10–22.
32. Lorenzo-Figueras, M.; Morisset, S.M.; Morisset, J.; Lainé, J.; Merrit, A.M. Digestive enzyme concentrations and activities in healthy pancreatic tissue of horses. *Am. J. Vet. Res.* 2007, *68*, 1070–1072.
33. Julliand, V.; De Fombelle, A.; Varloud, M. Starch digestion in horses: The impact of feed processing. *Livest. Sci.* 2006, *100*, 44–52.
34. Geor, R.J.; Acvim, D.; Harris, P.A.; Ecvcn, D. How to Minimize Gastrointestinal Disease Associated With Carbohydrate Nutrition in Horses. In Proceedings of the Proceedings in Annual Convention of the AAEP; 2007; pp. 178–185.
35. De Fombelle, A.; Varloud, M.; Goachet, A.G.; Jacotot, E.; Philippeau, C.; Drogoul, C.; Julliand, V. Characterization of the microbial and biochemical profile of the different segments of the digestive tract in horses given two distinct diets. *Anim. Sci.* 2003, *77*, 293–304.
36. De Fombelle, A.; Julliand, V.; Drogoul, C.; Jacotot, E. Feeding and microbial disorders in horses: 1-effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. *J. Equine Vet. Sci.* 2001, *21*, 439–445.
37. Richardson, K.; Murray, J.A.M.D. Fiber for Performance Horses: A Review. *J. Equine Vet. Sci.* 2016, *46*, 31–39.
38. Harris, P.A.; Ellis, A.D.; Fradinho, M.J.; Jansson, A.; Julliand, V.; Luthersson, N.; Santos, A.S.; Vervuert, I. Review: Feeding conserved forage to horses: Recent advances and recommendations. *Animal* 2017, *11*, 958–967.
39. Lindberg, J.E. Feedstuffs for horses. In *Equine Applied and Clinical Nutrition*; E-Book: Health, Welfare and Performance; Elsevier Ltd.: Amsterdam, The Netherlands, 2013; pp. 319–331.
40. Williamson, A.; Rogers, C.; Firth, E. A survey of feeding, management and faecal pH of Thoroughbred racehorses in the North Island of New Zealand. *N. Z. Vet. J.* 2011, *55*, 337–341.
41. Vanhonacker, F.; Verbeke, W.; Van Poucke, E.; Buijs, S.; Tuyttens, F.A.M. Societal concern related to stocking density, pen size and group size in farm animal production. *Livest. Sci.* 2009, *123*, 16–22.
42. Spinu, M.; Benveneste, S.; Degen, A.A. Effect of density and season on stress and behaviour in broiler breeder hens Effect of density and season on stress and behaviour in broiler breeder hens. *Br. Poult. Sci.* 2003, *44*, 170–174.
43. Fu, L.; Li, H.; Liang, T.; Zhou, B.; Chu, Q.; Schinckel, A.P.; Yang, X.; Zhao, R.; Li, P.; Huang, R. Stocking density affects welfare indicators of growing pigs of different group sizes after regrouping. *Appl. Anim. Behav. Sci.* 2016, *174*, 42–50.
44. Telezhenko, E.; von Keyserlingk, M.A.G.; Talebi, A.; Weary, D.M. Effect of pen size, group size,

and stocking density on activity in freestall-housed dairy cows. *J. Dairy Sci.* 2012, *95*, 3064–3069.

45. Veissier, I.; Butterworth, A.; Bock, B.; Roe, E. European approaches to ensure good animal welfare. *Appl. Anim. Behav. Sci. 113* 2008, *113*, 279–297.
46. Welfare Quality®. *Welfare Quality® Assessment Protocol for Pig (Sows and Piglets, Growing and Finishing Pigs)*; Welfare Quality®Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequality. net/media/1018/pig_protocol.pdf
47. Welfare Quality®. *Welfare Quality® Assessment Protocol for Poultry (Broilers and Laying Hens)*; Welfare Quality® Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequalitynetwork.net/ media/1019/poultry_protocol.pdf
48. Welfare Quality®. Welfare Quality® Assessment Protocol for Poultry (Broilers and Laying Hens); Welfare Quality® Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequalitynetwork.net/ media/1019/poultry_protocol.pdf d
49. The Swiss Federal Council. Animal Welfare Ordinance, 455.1. (English Version) 1–152. 2008. Available online: https://www.tierschutz.uzh.ch/dam/jcr:ffffffff-eb0a-0d29-ffff-ffff8b68fdea/animal_welfare_ordinance_ 455_1.pdf
50. Burla, J.B.; Rufener, C.; Bachmann, I.; Gygax, L.; Patt, A.; Hillmann, E. Space allowance of the littered area affects lying behavior in group-housed horses. *Front. Vet. Sci.* 2017, *4*, 1–12.
51. National Farm Animal Care Council. Code of Practice for the Care and Handling of Equines. Available online: https://www.nfacc.ca/pdfs/codes/equine_code_of_practice.pdf
52. Hartmann, E.; Søndergaard, E.; Keeling, L.J. Keeping horses in groups: A review. *Appl. Anim. Behav. Sci.* 2012, *136*, 77–87.
53. Benhajali, H.; Richard-Yris, M.-A.; Leroux, M.; Ezzaouia, M.; Charfi, F.; Hausberger, M. A note on the time budget and social behaviour of densely housed horses A case study in Arab breeding mares. *Appl. Anim. Behav. Sci.* 2008, *112*, 196–200.
54. Heleski, C.R.; Nielsen, B.D.; Zanella, A.J. Influence of housing on weanling horse behavior and subsequent welfare. *Appl. Anim. Behav. Sci.* 2002, *78*, 291–302.
55. Kogut, M.H.; Arsenault, R.J. Editorial: Gut Health: The New Paradigm in Food Animal Production. *Front. Vet. Sci.* 2016, *3*, 10–13.
56. Celi, P.; Cowieson, A.J.; Fru-Nji, F.; Steinert, R.E.; Kluenter, A.M.; Verlhac, V. Gastrointestinal functionality in animal nutrition and health: New opportunities for sustainable animal production. *Anim. Feed Sci. Technol.* 2017, *234*, 88–100.
57. Celi, P.; Verlhac, V.; Pérez Calvo, E.; Schmeisser, J.; Kluenter, A.-M. Biomarkers of gastrointestinal functionality in animal nutrition and health. *Anim. Feed Sci. Technol.* 2019, *250*, 9–31.
58. Colombino, E.; Prieto-Botella, D.; Capucchio, M.T. Gut health in veterinary medicine: A

bibliometric analysis of the literature. *Animals* 2021, *11*, 1–13.

59. Paun, A.; Danska, J.S. Immuno-ecology: How the microbiome regulates tolerance and autoimmunity. *Curr. Opin. Immunol.* 2015, *37*, 34–39.
60. Garber, A.; Hastie, P.; Murray, J.-A. Factors Influencing Equine Gut Microbiota: Current Knowledge. *J. Equine Vet. Sci.* 2020, *88*, 1–12.
61. Kraimi, N.; Dawkins, M.; Gebhardt-Henrich, S.G.; Velge, P.; Rychlik, I.; Volf, J.; Creach, P.; Smith, A.; Colles, F.; Leterrier, C. Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. *Physiol. Behav.* 2019, *210*, 112658.
62. Forder, R.E.A.; Howarth, G.S.; Tivey, D.R.; Hughes, R.J. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. *Poult. Sci.* 2007, *86*, 2396–2403.
63. Dowarah, R.; Verma, A.K.; Agarwal, N.; Patel, B.H.M.; Singh, P. Effect of swine based probiotic on performance, diarrhoea scores, intestinal microbiota and gut health of grower-finisher crossbred pigs. *Livest. Sci.* 2017, *195*, 74–79.
64. Biasato, I.; Ferrocino, I.; Biasibetti, E.; Grego, E.; Dabbou, S.; Sereno, A.; Gai, F.; Gasco, L.; Schiavone, A.; Cocolin, L.; et al. Modulation of intestinal microbiota, morphology and mucin composition by dietary insect meal inclusion in free-range chickens. *BMC Vet. Res.* 2018, *14*, 1–15.
65. Laudadio, V.; Passantino, L.; Perillo, A.; Lopresti, G.; Passantino, A.; Khan, R.U.; Tufarelli, V. Productive performance and histological features of intestinal mucosa of broiler chickens fed different dietary protein levels. *Poult. Sci.* 2012, *91*, 265–270.
66. Wambacq, W.A.; Van Doorn, D.A.; Rovers-Paap, P.M.; Ducatelle, R.; Vlaminck, L.; Lourenço, M.; Hesta, M. Dietary supplementation of micro-encapsulated sodium butyrate in healthy horses: effect on gut histology and immunohistochemistry parameters. *BMC Vet. Reseach* 2020, *16*, 1–9.
67. Pollitt, C.C.; Visser, M.B. Carbohydrate Alimentary Overload Laminitis. *Vet. Clin. North Am. - Equine Pract.* 2010, *26*, 65–78.
68. Geor, R.J.; Acvim, D. Metabolic Predispositions to Laminitis in Horses and Ponies: Obesity, Insulin Resistance and Metabolic Syndromes. *J. Equine Vet. Sci. 28*, 753–759.
69. Johnson, P.J.; Wiedmeyer, C.E.; LaCarrubba, A.; Ganjam, V.K.; Messer IV, N.T. Diabetes, Insulin Resistance, and Metabolic Syndrome in Horses Insulin Resistance in Horses: An Equine Metabolic Syndrome. *J. Diabetes Sci. Technol.* 2012, *6*, 1–7.
70. Bulmer, L.; McBride, S.; Williams, K.; Murray, J.A. The effects of a high-starch or high-fibre diet on equine reactivity and handling behaviour. *Appl. Anim. Behav. Sci.* 2015, *165*, 95–102.
71. Raspa, F.; Tarantola, M.; Bergero, D.; Nery, J.; Visconti, A.; Mastrazzo, C.M.; Cavallini, D.; Valvassori, E.; Valle, E. Time-Budget of Horses Reared for Meat Production : Influence of Stocking

Density on Behavioural Activities and Subsequent Welfare. *Animals* 2020, *10*, 1334.

72. Hothersall, B.; Nicol, C. Role of Diet and Feeding in Normal and Stereotypic Behaviors in Horses. *Vet. Clin. North Am. Equine Pract.* 2009, *25*, 167–181.
73. Murray, J.-A.M.D.; Bloxham, C.; Kulifay, J.; Stevenson, A.; Roberts, J. Equine Nutrition: A Survey of Perceptions and Practices of Horse Owners Undertaking a Massive Open Online Course in Equine Nutrition. *J. Equine Vet. Sci.* 2015, *35*, 510–517.
74. Hoffman, C.J.; Costa, L.R.; Freeman, L.M. Survey of Feeding Practices, Supplement Use, and Knowledge of Equine Nutrition among a Subpopulation of Horse Owners in New England. *J. Equine Vet. Sci.* 2009, *29*, 719–726.
75. Raspa, F.; Tarantola, M.; Bergero, D.; Bellino, C.; Mastrazzo, C.M.; Visconti, A.; Valvassori, E.; Vervuert, I.; Valle, E. Stocking Density Affects Welfare Indicators in Horses Reared for Meat Production. *Animals* 2020, *10*, 1103.
76. Davis, J.L.; Blikslager, A.T.; Catto, K.; Jones, S.L. A Retrospective Analysis of Hepatic Injury in Horses with Proximal Enteritis (1984-2002). *J. Vet. Intern. Med.* 2003, *17*, 896–901.
77. Grimm, P.; Philippeau, C.; Julliand, V. Faecal parameters as biomarkers of the equine hindgut microbial ecosystem under dietary change. *Animal* 2017, *11*, 1136–1145.
78. Ericsson, A.C.; Johnson, P.J.; Lopes, M.A.; Perry, S.C.; Lanter, H.R. A microbiological map of the healthy equine gastrointestinal tract. *PLoS One* 2016, *11*, 1–17.
79. Costa, M.C.; Silva, G.; Ramos, R. V; Staempfli, H.R.; Arroyo, L.G.; Kim, P.; Weese, J.S. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. *Vet. J.* 2015, *205*, 74–80.
80. Fraser, D.; Weary, D.M.; Pajor, E.A.; Milligan, B.N. A Scientific Conception of Animal Welfare that Reflects Ethical Concerns. *Anim. Welf.* 1997, *6*, 187–205.
81. Dawkins, M.S. Behaviour as a tool in the assessment of animal welfare. *Zoology* 2003, *106*, 383–387.
82. Waran, N.K. Can studies of feral horse behaviour be used for assessing domestic horse welfare? *Equine Vet. J.* 1997, *29*, 249–251.
83. Goodwin, D. The importance of ethology in understanding the behaviour of the horse. *Equine Vet. J. Suppl.* 1999, *28*, 15–19.
84. Lesimple, C.; Poissonnet, A.; Hausberger, M. How to keep your horse safe? An epidemiological study about management practices. *Appl. Anim. Behav. Sci.* 2016, *181*, 105–114.
85. McGreevy, P. Ingestive behaviour. In Equine Behavior: A Guide for Veterinarians and Equine Scientist; Saunders Ltd.: Nottingham, UK, 2004; pp. 189–215.
86. Wageningen UR Livestock Research Welfare Monitoring System - Assessment protocol for horses Available online: http://edepot.wur.nl/238619
87. Carroll, C.L.; Huntington, P.J. Body condition scoring and weight estimation of horses. *Equine*

Vet. J. 1988, *20*, 41–45.

88. Fraser, D.; Duncan, I.J.H.; Edwards, S.A.; Grandin, T.; Gregory, N.G.; Guyonnet, V.; Hemsworth, P.H.; Huertas, S.M.; Huzzey, J.M.; Mellor, D.J.; et al. General Principles for the welfare of animals in production systems: The underlying science and its application. *Vet. J.* 2013, *198*, 19–27.
89. McFarland, D.J.; Sibly, R.M. The Behavioural Final Common Path. Philosophical Transactions of the Royal Society of London. *R. Soc. Stable* 1975, 270, 265. Available online: https://www.jstor.org/stable/2417384
90. Martin, P.; Bateson, P. Recording methods. In *Measuring Behaviour: An Introductory Guide*, 2nd ed.; Cambridge University Press: Cambridge, UK, 1993; pp. 84–100.
91. Altmann, J. Observational Study of Behavior: Sampling Methods. *Behaviour* 1974, *49*, 227–266.
92. Puth, M.T.; Neuhäuser, M.; Ruxton, G.D. Effective use of Pearson's product-moment correlation coefficient. *Anim. Behav.* 2014, *93*, 183–189.
93. Prion, S.; Haerling, K.A. Making sense of methods and measurement: Pearson product-moment correlation coefficient. *Clin. Simul. Nurs.* 2014, *10*, 587–588.
94. Boyd, L.E.; Carbonaro, D.A.; Houpt, K.A. The 24-Hour Time Budget of Przewalski Horses. *Appl. Anim. Behav. Sci.* 1988, *21*, 5–17.
95. Raspa, F.; Dinardo, F.R.; Vervuert, I.; Bergero, D.; Bottero, M.T.; Pattono, D.; Dalmasso, A.; Vinassa, M.; Valvassori, E.; Bruno, E.; et al. A Fibre- vs. cereal grain-based diet: Which is better for horse welfare? Effects on intestinal permeability, muscle characteristics and oxidative status in horses reared for meat production. *J. Anim. Physiol. Anim. Nutr. (Berl).* 2021, *00*, 1–14.
96. Vondran, S.; Venner, M.; Coenen, M.; Vervuert, I. Effects of alfalfa chaff on the gastric mucosa in adult horses. *Pferdeheilkunde* 2017, *33*, 66–71.
97. Naumann C, Bassler R, Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik: (Methodenbuch). 3rd ed. Darmstadt: VDLUFA-Verl.; 2004.
98. Guantario, B.; Giribaldi, M.; Devirgiliis, C.; Finamore, A.; Colombino, E.; Capucchio, M.T.; Evangelista, R.; Motta, V.; Zinno, P.; Cirrincione, S.; et al. A comprehensive evaluation of the impact of bovine milk containing different beta-casein profiles on gut health of ageing mice. *Nutrients* 2020, *12*, 1–19.
99. Ząbek, K.; Szkopek, D.; Michalczuk, M.; Konieczka, P. Dietary phytogenic combination with hops and a mixture of a free butyrate acidifier and gluconic acid maintaining the health status of the gut and performance in chickens. *Animals* 2020, *10*, 1–12.
100. Webb, H.E.; Brichta-Harhay, D.M.; Brashears, M.M.; Nightingale, K.K.; Arthur, T.M.; Bosilevac, J.M.; Kalchayanand, N.; Schmidt, J.W.; Wang, R.; Granier, S.A.; et al. Salmonella in peripheral lymph nodes of healthy cattle at Slaughter. *Front. Microbiol.* 2017, *8*, 1–10.
101. Mainar-Jaime, R.C.; Andrés, S.; Vico, J.P.; San Román, B.; Garrido, V.; Grilló, M.J. Sensitivity of the ISO 6579:2002/Amd 1:2007 standard method for detection of *Salmonella* spp. on mesenteric

lymph nodes from slaughter pigs. *J. Clin. Microbiol.* 2013, *51*, 89–94.

102. Cavallini, D.; Mammi, L.M.E.; Buonaiuto, G.; Palmonari, A.; Valle, E.; Formigoni, A. Immune-metabolic-inflammatory markers in Holstein cows exposed to a nutritional and environmental stressing challenge. *J. Anim. Physiol. Anim. Nutr. (Berl).* 2021, *105*, 42–55.
103. De Palo, P.; Maggiolino, A.; Centoducati, N.; Tateo, A. Effects of different milk replacers on carcass traits, meat quality, meat color and fatty acids profile of dairy goat kids. *Small Rumin. Res.* 2015, *131*, 6–11.
104. De Palo, P.; Tateo, A.; Maggiolino, A.; Marino, R.; Ceci, E.; Nisi, A.; Lorenzo, J.M. Martina Franca donkey meat quality: Influence of slaughter age and suckling technique. *Meat Sci.* 2017, *134*, 128–134.
105. De Palo, P.; Maggiolino, A.; Centoducati, P.; Tateo, A. Colour changes in meat of foals as affected by slaughtering age and post-thawing time. *Asian-Australasian J. Anim. Sci.* 2012, *25*, 1775–1779.
106. De Palo, P.; Tateo, A.; Maggiolino, A.; Centoducati, P. Effect of nutritive level on carcass traits and meat quality of IHDH foals. *Anim. Sci. J.* 2014, *85*, 780–786.
107. Hornsey, H.C. The colour of cooked cured pork. I.—Estimation of the Nitric oxide-Haem Pigments. *J. Sci. Food Agric.* 1956, *7*, 534–540.
108. De Palo, P.; Maggiolino, A.; Centoducati, P.; Milella, P.; Calzaretti, G.; Tateo, A. Is meat quality from Longissimus lumborum samples correlated with other cuts in horse meat? *Anim. Sci. J.* 2016, *87*, 428–438.
109. Tufarelli, V.; Laudadio, V.; Casalino, E. An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in meat-type broiler chickens. *Environ. Sci. Pollut. Res.* 2016, *23*, 6197–6204.
110. Tateo, A.; Maggiolino, A.; Domínguez, R.; Lorenzo, J.M.; Dinardo, F.R.; Ceci, E.; Marino, R.; della Malva, A.; Bragaglio, A.; De Palo, P. Volatile organic compounds, oxidative and sensory patterns of vacuum aged foal meat. *Animals* 2020, *10*, 1–17.
111. De Palo, P.; Maggiolino, A.; Albenzio, M.; Casalino, E.; Neglia, G.; Centoducati, G.; Tateo, A. Survey of biochemical and oxidative profile in donkey foals suckled with one natural and one semi-artificial technique. *PLoS One* 2018, *13*, e0198774.
112. Gondim, F.J.; Zoppi, C.C.; dos Reis Silveira, L.; Pereira-da-Silva, L.; Vaz de Macedo, D. Possible Relationship Between Performance and Oxidative Stress in Endurance Horses. *J. Equine Vet. Sci.* 2009, *29*, 206–212.
113. Faure, P.; Lafond, J.L. Measurement of plasma sulfhydryl and carbonyl groups as a possible indicator of protein oxidation. In *Analysis of Free Radicals in Biological Systems*; Birkhäuser Basel, 1995; pp. 237–248.
114. Södergren, E.; Nourooz-Zadeh, J.; Berglund, L.; Vessby, B. Re-evaluation of the ferrous oxidation

in xylenol orange assay for the measurement of plasma lipid hydroperoxides. *J. Biochem. Biophys. Methods* 1998, *37*, 137–146.

115. Maggiolino, A.; Lorenzo, J.M.; Salzano, A.; Faccia, M.; Blando, F.; Serrano, M.P.; Latorre, M.A.; Quiñones, J.; De Palo, P. Effects of aging and dietary supplementation with polyphenols from Pinus taeda hydrolysed lignin on quality parameters, fatty acid profile and oxidative stability of beef Animal Production Science. *Anim. Prod. Sci.* 2020, *60*, 713–724.
116. De Palo, P.; Maggiolino, A.; Tateo, A.; Centoducati, P. Influence of gas mixture on quality and shelf life of veal calf meat. *Ital. J. Anim. Sci.* 2014, *13*, 226–233.
117. De Palo, P.; Maggiolino, A.; Centoducati, P.; Tateo, A. Effects of two different packaging materials on veal calf meat quality and shelf life. *J. Anim. Sci.* 2013, *91*, 2920–2930.
118. Landis, J.R.; Koch, G.G. The Measurement of Observer Agreement for Categorical Data. *Biometrics* 1977, *33*, 159.
119. Blanco-Penedo, I.; Ló Pez-Alonso, M.; Shore, R.F.; Miranda, M.; Castillo, C.; Herná Ndez, J.; Benedito, J.L. Evaluation of organic, conventional and intensive beef farm systems: health, management and animal production. *Animal* 2012, *6*, 1503–1511.
120. Viksten, S.M.; Visser, E.K.; Blokhuis, H.J. A comparative study of the application of two horse welfare assessment protocols. *Acta Agric. Scand. A Anim. Sci.* 2016, *66*, 56–65.
121. Webster, J. Critical control points in the delivery of improved animal welfare. *Anim. Welf.* 2012, *21*, 117–123.
122. Dalla Costa, E.; Murray, L.; Dai, F.; Canali, E.; Minero, M. Equine on-farm welfare assessment: A review of animal-based indicators. *Anim. Welf.* 2014, *23*, 323–341.
123. Jørgensen, G.H.M.; Borsheim, L.; Mejdell, C.M.; Søndergaard, E.; Bøe, K.E. Grouping horses according to gender-Effects on aggression, spacing and injuries. *Appl. Anim. Behav. Sci.* 2009, *120*, 94–99.
124. Ninomiya, S.; Kusunose, R.; Sato, S.; Terada, M.; Sugawara, K. Effects of feeding methods on eating frustration in stabled horses. *Anim. Sci. J.* 2004, *75*, 465–469.
125. Hammer, C.; Holzbauer, S. Equine Biological Risk Management. Iowa State University Center for Food Security and Public Health Web Site. Available online: www.cfsph.iastate.edu/BRM.
126. Dugdale, A.H.A.; Grove-White, D.; Curtis, G.C.; Harris, P.A.; Argo, C.M.C.G. Body condition scoring as a predictor of body fat in horses and ponies. *Vet. J.* 2012, *194*, 173–178.
127. Yarnell, K.; Hall, C.; Royle, C.; Walker, S.L. Domesticated horses differ in their behavioural and physiological responses to isolated and group housing. *Physiol. Behav.* 2015, *143*, 51–57.
128. Lesimple, C. Indicators of Horse Welfare: State-of-the-Art. *Animals* 2020, *10*, 294.
129. Pritchard, J.C.; Lindberg, A.C.; Main, D.C.J.; Whay, H.R. Assessment of the welfare of working horses, mules and donkeys, using health and behaviour parameters. *Prev. Vet. Med.* 2005, *69*, 265–283.

130. Burn, C.C.; Dennison, T.L.; Whay, H.R. Relationships between behaviour and health in working horses, donkeys, and mules in developing countries. *Appl. Anim. Behav. Sci.* 2010, *126*, 109–118.
131. Fureix, C.; Jego, P.; Henry, S.; Lansade, L.; Hausberger, M. Towards an Ethological Animal Model of Depression? A Study on Horses. *PLoS One* 2012, *7*, e39280.
132. Duncan, P. Time-budgets of Camargue horses: II. Time-budgets of adult horses and weaned sub-adults. *Bahaviour* 1980, *72*, 26–49.
133. Yarnell, K.; Hall, C.; Royle, C.; Walker, S.L. Domesticated horses differ in their behavioural and physiological responses to isolated and group housing. *Physiol. Behav.* 2015, *143*, 51–57.
134. Huzzey, J.M.; DeVries, T.J.; Valois, P.; von Keyserlingk, M.A.G. Stocking Density and Feed Barrier Design Affect the Feeding and Social Behavior of Dairy Cattle. *J. Dairy Sci.* 2006, *89*, 126–133.
135. Weeks, C.A. A review of welfare in cattle, sheep and pig lairages, with emphasis on stocking rates, ventilation and noise. *Anim. Welf.* 2008, *17*, 275–284.
136. Vasseur, E.; Rushen, J.; Haley, D.B.; de Passillé, A.M. Sampling cows to assess lying time for on-farm animal welfare assessment. *J. Dairy Sci.* 2012, *95*, 4968–4977.
137. Chaplin, S.J.; Gretgrix, L. Effect of housing conditions on activity and lying behaviour of horses. *Animal* 2010, *4*, 792–795.
138. Jensen, M.B.; Vestergaard, K.S.; Krohn, C.C. Play behaviour in dairy calves kept in pens: the effect of social contact and space allowance Margit. *Appl. Anim. Behav. Sci.* 1998, *56*, 97–108.
139. Cornale, P.; Macchi, E.; Miretti, S.; Renna, M.; Lussiana, C.; Perona, G.; Mimosi, A. Non-canine Research Effects of stocking density and environmental enrichment on behavior and fecal corticosteroid levels of pigs under commercial farm conditions. *J. Vet. Behav. 10* 2015, *10*, 569–576.
140. Lesimple, C.; Fureix, C.; Lescolan, N.; Richard-Yris, M.-A.; Hausberger, M. Housing conditions and breed are associated with emotionality and cognitive abilities in riding school horses. *Appl. Anim. Behav. Sci.* 2011, *129*, 92–99.
141. Boissy, A.; Manteuffel, G.; Jensen, M.B.; Moe, R.O.; Spruijt, B.; Keeling, L.J.; Winckler, C.; Forkman, B.; Dimitrov, I.; Langbein, J.; et al. Assessment of positive emotions in animals to improve their welfare. *Physiol. Behav.* 2007, *92*, 375–397.
142. Napolitano, F.; Knierim, U.; Grass, F.; De Rosa, G. Positive indicators of cattle welfare and their applicability to on-farm protocols. *Ital. J. Anim. Sci.* 2009, *8*, 355–365.
143. Held, S.D.E.; Spinka, M. Animal play and animal welfare. *Anim. Behav.* 2011, *81*, 891–899.
144. Blois-Heulin, C.; Rochais, C.; Camus, S.; Fureix, C.; Lemasson, A.; Lunel, C.; Bezard, E.; Hausberger, M. Animal welfare: Could adult play be a false friend? *Anim. Behav. Cogn.* 2015, *2*, 156–185.
145. Goodwin, D. The importance of ethology in understanding the behaviour of the horse. Equine

Vet. J. Suppl. 1999, 28, 15–19.

146. Cooper, J.J.; Albentosa, M.J. Behavioural adaptation in the domestic horse: potential role of apparently abnormal responses including stereotypic behaviour. *Livest. Prod. Sci.* 2005, *92*, 177–182.
147. Lopes, M.A.F.; White, N.A.; Crisman, M. V.; Ward, D.L. Effects of feeding large amounts of grain on colonic contents and feces in horses. *Am. J. Vet. Res.* 2004, *65*, 687–694.
148. Sadet-Bourgeteau, S.; Julliand, V.; Philippeau, C.; Julliand, V. Effect of concentrate feeding sequence on equine hindgut fermentation parameters Isolation and characterisation of equine fibrolytic bacteria View project Effect of concentrate feeding sequence on equine hindgut fermentation parameters. *Animal* 2016, *11*, 1146–1152.
149. Medina, B.; Girard, I.D.; Jacotot, E.; Julliand, V. Effect of a preparation of Saccharomyces cerevisiae on microbial profiles and fermentation patterns in the large intestine of horses fed a high fiber or a high starch diet 1. *J. Anim. Sci* 2002, 2600–2609.
150. Dougal, K.; de la Fuente, G.; Harris, P.A.; Girdwood, S.E.; Pinloche, E.; Geor, R.J.; Nielsen, B.D.; Schott, H.C.; Elzinga, S.; Newbold, C.J. Characterisation of the Faecal Bacterial Community in Adult and Elderly Horses Fed a High Fibre, High Oil or High Starch Diet Using 454 Pyrosequencing. *PLoS One* 2014, *9*, e87424.
151. Nadeau, J.A.; Andrews, F.; Patton, C.S.; Argenzio, R.A.; Mathew, A.G.; Saxton, A.M. Effects of hvdrochloric, valeric, and other volatile fatty acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses. *Artic. Am. J. Vet. Res.* 2003, *64*, 413–417.
152. Kim, Y.S.; Unno, T.; Kim, B.Y.; Park, M.S. Sex differences in gut microbiota. *World J. Mens. Health* 2020, *38*, 48–60.
153. Kauter, A.; Epping, L.; Semmler, T.; Antao, E.-M.; Kannapin, D.; Stoeckle, S.D.; Gehlen, H.; Lübke-Becker, A.; Günther, S.; Wieler, L.H.; et al. The gut microbiome of horses: current research on equine enteral microbiota and future perspectives. *Anim. Microbiome* 2019, 1–14.
154. Lambert, G.P. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J. Anim. Sci.* 2009, *87*, 101–108.
155. National Research Council (NRC). Nutrient Requirements for Horses. 6th edition. National Academies Press: Washington, DC, USA; 2007.
156. Ellis, A. Energy systems and requirements; In *Equine Applied and Clinical Nutrition*; E-Book: Health, Welfare and Performance; Elsevier Ltd.: Amsterdam, The Netherlands, 2013; pp. 96-112.
157. Smolders, E.A.A.; Steg, A.; Hindle, V.A. Organic matter digestibility in horses and its prediction. *Netherlands J. Agric. Sci.* 1990, *38*, 435–447.
158. Dabareiner, R.M.; White, N.A. Diseases and surgery of the cecum. *Vet. Clin. North Am. Equine Pract.* 1997, *13*, 303–315.

159. White, N.A.; Dabareiner, R.M. Treatment of impaction colics. *Vet. Clin. North Am. Equine Pract.* 1997, *13*, 243–259.
160. Hummel, J.; Scheurich, F.; Ortmann, S.; Crompton, L.A.; Gerken, M.; Clauss, M. Comparative selective retention of particle size classes in the gastrointestinal tract of ponies and goats. *J. Anim. Physiol. Anim. Nutr. (Berl).* 2018, *102*, 429–439.
161. Gunnarsdottir, H.; Van der Stede, Y.; De Vlamynck, C.; Muurling, F.; De Clercq, D.; van Loon, G.; Vlaminck, L. Hospital-based study of dental pathology and faecal particle size distribution in horses with large colon impaction. *Vet. J.* 2014, *202*, 153–156.
162. Ralston, S.L.; Foster, D.L.; Divers, T.; Hintz, H.F. Effect of dental correction on feed digestibility in horses. *Equine Vet. J.* 2001, *33*, 390–393.
163. Zwirglmaier, S.; Remler, H.P.; Senckenberg, E.; Fritz, J.; Stelzer, P.; Kienzle, E. Effect of dental correction on voluntary hay intake, apparent digestibility of feed and faecal particle size in horse. *J. Anim. Physiol. Anim. Nutr. (Berl).* 2013, *97*, 72–79.
164. Julliand, V.; Grimm, P. The Impact of Diet on the Hindgut Microbiome. *J. Equine Vet. Sci.* 2017, *52*, 23–28.
165. Duvnjak, M.; Bošnjak, A.; Zadravec, M.; Pintar, J. Starch in horse diet improves feces microbiota, in vitro digestibility of fiber. *J. Cent. Eur. Agric.* 2018, *19*, 918–930.
166. Benhajali, H.; Richard-Yris, M.-A.; Ezzaouia, M.; Charfi, F.; Hausberger, M. Foraging opportunity: a crucial criterion for horse welfare? *Animal* 2009, *3*.
167. Houpt, K.A.; Houpt, T.R. Social and Illumination Preferences of Mares; *J. Anim. Sci.* 1988; 66, 2159–2164.
168. Hausberger, M.; Fureix, C.; Bourjarde, M.; Wessel-Robert, S.; Richard-Yris, M.A. On the significance of adult play: what does social play tell us about adult horse welfare? *Naturwissenschaften* 2012, *99*, 291–302.
169. Mcdonnell, S.M.; Poulin, A. Equid play ethogram. *Appl. Anim. Behav. Sci.* 2002, *78*, 263–290.
170. Knubben, J.M.; Fürst, A.; Gygax, L.; Stauffacher, M. Bite and kick injuries in horses: Prevalence, risk factors and prevention. *Equine Vet. J.* 2008, *40*, 219–223.
171. Mills, D.S.; Nankervis, K.J. Sexual and reproductive behaviour in horses. In *Equine Behaviour: Principles and Practice*; Blackwell Science Ltd., Ed.; 1999; pp. 139–160.
172. Keiper, R.R.; Sambraus, H.H. The stability of equine dominance hierarchies and the effects of kinship, proximity and foaling status on hierarchy rank. *Appl. Anim. Behav. Sci.* 1986, *16*, 121–130.
173. Seong, P.N.; Seo, H.W.; Kim, J.-H.; Kang, G.H.; Cho, S.-H.; Chae, H.S.; Park, B.Y.; Van Ba, H. Assessment of frozen storage duration effect on quality characteristics of various horse muscles. *Asian-Australasian J. Anim. Sci.* 2017, *30*, 1756–1763.
174. Lawrie, R.A. The onset of rigor mortis in various muscles of the draught horse. *J. Physiol.* 1953,

121, 275–288.

175. Muir, P.D.; Deaker, J.M.; Bown, M.D.; Muir J M Deaker M D Bown, P.D. Effects of forage-and grain-based feeding systems on beef quality: A review. *New Zeal. J. Agric. Res.* 1998, *41*, 623–635.
176. Martin- Rosset, W. The growing horse. In *Equine Nutrition: INRA nutrient requirements, recommended allowances and feed tables.* Academic Publishers, 2015; pp. 169– 215.
177. Mancini, R.A.; Hunt, M.C. Current research in meat color. *Meat Sci.* 2005, *71*, 100–121.
178. Franco, D.; Crecente, S.; Antonio Vázquez, J.; Gómez, M.; Lorenzo, J.M. Effect of cross breeding and amount of finishing diet on growth parameters, carcass and meat composition of foals slaughtered at 15 months of age. *Meat Sci.* 2013, *93*, 547–556.
179. Juárez, M.; Polvillo, O.; Alcalde, M.; Romero, F.; Valera, M. Breed effect on carcass and meat quality of foals slaughtered at 24 months of age. *Meat Sci.* 2009, *83*, 224–228.
180. Carrillo, J.A.; He, Y.; Li, Y.; Liu, J.; Erdman, R.A.; Sonstegard, T.S.; Song, J. Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare. *Sci. Rep.* 2016, *6*.
181. Reagan, J.O.; Carpenter, J.A.; Bauer, F.T.; Lowrey, R.S. Packaging and palatability characteristics of grass and grass-grain beef. *J. Anim. Sci.* 1977, *46*, 716–721.
182. Duckett, S.K.; Wagner, D.G.; Yates, L.D.; Dolezal, H.G.; May2, S.G. Effects of Time on Feed on Beef Nutrient Composition'. *J. Anim. Sci.* 1993, *71*, 2079–2088.
183. Urschel, K.L.; Lawrence, L.M. Amino acids and protein. In *Equine Applied and Clinical Nutrition: Health, Welfare and Performance*; E-Book: Health, Welfare and Performance; Elsevier Ltd.: Amsterdam, The Netherlands, 2013; pp. 113–135.
184. Mahecha, L.; Nuernberg, K.; Nuernberg, G.; Ender, K.; Hagemann, E.; Dannenberger, D. Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls. *Meat Sci.* 2009, *82*, 365–371.
185. Avellini, L.; Chiaradia, E.; Gaiti, A. Effect of exercise training, selenium and vitamin E on some free radical scavengers in horses (Equus caballus). *Comp. Biochem. Physiol.* 1999, *123*, 147–154.
186. Cardenia, V.; Rodriguez-Estrada, M.T.; Cumella, F.; Sardi, L.; Della Casa, G.; Lercker, G. Oxidative stability of pork meat lipids as related to high-oleic sunflower oil and vitamin E diet supplementation and storage conditions. *Meat Sci.* 2011, *88*, 271–279.
187. Voljč, M.; Frankič, T.; Levart, A.; Nemec, M.; Salobir, J. Evaluation of different vitamin e recommendations and bioactivity of α-tocopherol isomers in broiler nutrition by measuring oxidative stress in vivo and the oxidative stability of meat. *Poult. Sci.* 2011, *90*, 1478–1488.
188. Cappai, M.G.; Pudda, F.; Wolf, P.; Accioni, F.; Boatto, G.; Pinna, W. Variation of Hematochemical Profile and Vitamin E Status in Feral Giara Horses From Free Grazing in the Wild to Hay Feeding

During Captivity. *J. Equine Vet. Sci.* 2020, *94*, 103220.

189. Cappai, M.G.; Taras, A.; Cossu, I.; Cherchi, R.; Dimauro, C.; Accioni, F.; Boatto, G.; Deroma, M.; Spanu, E.; Gatta, D.; et al. Effects of Dietary Zn/Se and α-Tocopherol Supplementation on Metabolic Milieu, Haemogram and Semen Traits of Breeding Stallions. *Biol. Trace Elem. Res.* 2020, 1–10.
190. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J. Med.* 2018, *54*, 287–293.
191. Fang, T.; Wu, X.; Cao, W.; Jia, G.; Zhao, H.; Chen, X.; Wu, C.; Tang, J.; Wang, J.; Liu, G. Effects of dietary fiber on the antioxidant capacity, immune status, and antioxidant-relative signaling molecular gene expression in rat organs. *RSC Adv.* 2017, *7*, 19611–19620.

8. RESEARCH PRODUCTS

8.1. Articles related to the PhD project

1. Raspa, F.; Dinardo, F.R.; Vervuert, I.; Bergero, D.; Bottero, M.T.; Pattono, D.; Dalmasso, A.; Vinassa, M.; Valvassori, E.; Bruno, E., De Palo, P.; Valle, E. A Fibre- vs. cereal grain-based diet: Which is better for horse welfare? Effects on intestinal permeability, muscle characteristics and oxidative status in horses reared for meat production. Journal of Animal Physiology and Animal Nutrition. 2021, *00*, 1–14

2. Raspa, F.; Tarantola, M.; Bergero, D.; Bellino, C.; Mastrazzo, C.M.; Visconti, A.; Valvassori, E.; Vervuert, I.; Valle, E. 2020. Stocking Density Affects Welfare Indicators in Horses Reared for Meat Production. Animals., 2020, 10, 1103

3. Raspa, F.; Tarantola, M.; Bergero, D.; Nery, J.; Visconti, A.; Mastrazzo, C.M.; Cavallini, D.; Valvassori, E.; Valle, E. 2020. Time-Budget of Horses Reared for Meat Production: Influence of Stocking Density on Behavioural Activities and Subsequent Welfare. Animals., 2020, 10, 1334

8.2. Articles not related to the PhD project

4. Raspa, F.; Roggero, A.; Palestrini, C.; Canavesio, M.M.; Bergero, D.; Valle, E. Studying the Shape Variations of the Back, the Neck, and the Mandibular Angle of Horses Depending on Specific Feeding Postures Using Geometric Morphometrics. Animals., 2021, 1–15

5. Tassone, S.; Fortina, R.; Valle, E.; Cavallarin, L.; Raspa, F.; Boggero, S.; Bergero, D.; Giammarino, M.; Renna, M. Comparison of In Vivo and In Vitro Digestibility in Donkeys. Animals., 2020, 10, 2100

6. Vinassa, M.; Cavallini, D.; Galaverna, D.; Baragli P.; Raspa F.; Nery, J.; Valle, E. Palatability assessment in horses in relation to lateralization and temperament. Applied animal behaviour science., 2020, 232

7. Raspa, F.; Cavallarin, L.; McLean, A. K.; Bergero, D.; Valle, E. A Review of the Appropriate Nutrition Welfare Criteria of Dairy Donkeys: Nutritional Requirements, Farm Management Requirements and Animal-Based Indicators. Animals., 2019, 9, 315

8.3. Abstracts on international congress related to the PhD project

1. Raspa F., Tarantola M., Bergero D., Valvassori E., Mastrazzo C.M., Visconti A., Valle E., 2019. A preliminary study on the behaviours of horses reared for meat production: effect of the space available on daily activity budget. 23th Congress of the European Society of Veterinary and Comparative Nutrition, Torino, 18-20 settembre, 89 p.

2. Raspa F., Tarantola M., Bergero D., Bellino C., Valvassori E., Mastrazzo C.M., Visconti A., Valle E., 2019. A preliminary study on the welfare of horses reared for meat production: how the space available affects the welfare parameters of appropriate nutrition. 23th Congress of the European Society of Veterinary and Comparative Nutrition, Torino, 18-20 settembre, 90 p.

3. Raspa F., Cavallini D., Vervuert I., Valvassori E., Mammi L.M.E., Bergero D. Valle E. 2020. Impact of two different diets on faecal parameters of horses. 24th Congress of the European Society of Veterinary and Comparative Nutrition On line 17-20settembre, 96 p.

4. Raspa F., De Palo P., Vervuert I., Bergero D., Valvassori E., Valle E. 2020. Antioxidant enzymes and oxidative stress end products in horses fed with different feeding strategie. 71th EAAP Virtual Annual Meeting 1-4 dicembre, Theatre session 39, 415 p.

5. Raspa F., Vervuert I., De Palo P., Cavallini D., Bergero D., Valvassori E., Valle E. 2021. Influence of two feeding managements on behaviour and welfare in horses reared for meat production. 72th EAAP Annual Meeting, Davos (Switzerland), 30 agosto-3 settembre, Theatre session 28, 308 p.

6. Raspa F., Colombino E., Capucchio M.T., Cavallini D., Vervuert I., Bottero M.T., Pattono D., Dalmasso A., Bergero D., Valvassori E., Valle E. 2021. Effects of feeding managements on microbial contamination of mesenteric lymph nodes and liver and on intestinal histo-morphology in horses. 25th Congress of the European Society of Veterinary and Comparative Nutrition On line 9-11 settembre, 93 p.

8.4. Abstracts on international congress not related to the PhD project

7. Raspa F., Valle E., Bergero D., Addamo G., Paonessa F., Virone G., 2019. A preliminary study on the feasibility of using microwave frequencies to determine several tissue samples from horses. 23th Congress of the European Society of Veterinary and Comparative Nutrition, Torino, 18-20 settembre, 169 p.

8. Cavallini D., Pagliara E., Raspa F., Valle E., 2019. Cases of stomatitis induced by Setaria glauca and Echinochloa crus-galli in horses. 23th Congress of the European Society of Veterinary and Comparative Nutrition, Torino, 18-20 settembre, 171 p.

9. Raspa F., Vergnano D., Cavallarin L., Mclean A., Tarantola M., Valle E., 2019. Meeting nutritional needs to ensure dairy donkeys' welfare. 70th EAAP Annual Meeting, Ghent, 26-30 agosto.

8.5. Awards

EAAP (European Federation of Animal Science) Scholarship Winner within the 72nd EAAP Annual Meeting, Davos, Switzerland – Title: "Influence of two feeding managements on behaviour and welfare in horses reared for meat production"

8.6. Stays abroad

1/02/2020 – 16/03/2020: Institute of Animal Nutrition, Nutrition Diseases and Dietetics, Faculty of Veterinary Medicine, Leipzig University under the supervision of Professor Ingrid Vervuert.

animals

MDPI

Article

Stocking Density Affects Welfare Indicators in Horses Reared for Meat Production

Federica Raspa [1,*], Martina Tarantola [1], Domenico Bergero [1], Claudio Bellino [1], Chiara Maria Mastrazzo [1], Alice Visconti [1], Ermenegildo Valvassori [2], Ingrid Vervuert [3] and Emanuela Valle [1]

1 Department of Veterinary Sciences, University of Turin, 10095 Grugliasco, Italy; martina.tarantola@unito.it (M.T.); domenico.bergero@unito.it (D.B.); claudio.bellino@unito.it (C.B.); chiara.mastrazzo@edu.unito.it (C.M.M.); alice.visconti@edu.unito.it (A.V.); emanuela.valle@unito.it (E.V.)
2 Public Veterinary Service, ASL TO5 Piedmont, Italy; e.valvassori@tin.it
3 Institute of Animal Nutrition, Nutrition Diseases and Dietetics, Faculty of Veterinary Medicine, Leipzig University, 04103 Leipzig, Germany; ingrid.vervuert@vetmed.uni-leipzig.de
* Correspondence: federica.raspa@unito.it

Received: 9 March 2020; Accepted: 23 June 2020; Published: 26 June 2020

Simple Summary: Not enough effort is being made to safeguard the welfare of horses reared for meat production. These horses are kept in intensive breeding farms where they are housed in group pens at high stock densities and fed high amounts of concentrates. The aim of the study is to evaluate whether the stocking density of horses raised in group pens for meat production and their feeding management affects their welfare according to different stocking density. According to our results, when the horses had more than 4.75 m^2/horse, many indicators were affected (i.e., improvement of coat cleanliness, improvement of bedding quantity, improvement of mane and tail conditions, less resting in a standing position, and less feeding related to the greater space available at the feed bunk). However, a further increment of space and/or changes in management regimes may be necessary to improve all the welfare indicators. The results also revealed the need to improve the feeding management of these animals.

Abstract: Horses kept for meat production are reared in intensive breeding farms. We employed a checklist adapted from the Animal Welfare Indicators (AWIN) assessment protocol. Our evaluation aims to assess whether welfare indicators are influenced by stocking densities (m^2/horse) and feeding strategies applied. An analysis was carried out on the data obtained from 7 surveys conducted at a single horse farm designed for meat production. In each survey, the same 12 pens were assessed, but on each occasion, the horses in the pens had been changed as had the stocking densities. Briefly, 561 horses aged 16 ± 8 months (mean ± standard deviation) were evaluated. Two stocking density cut-off values (median and 75th percentile: 3.95 and 4.75 m^2/horse, respectively) were applied to investigate the effect of stocking density on horse welfare. Data were analysed using Mann–Whitney U and Fisher's exact tests ($p < 0.05$). When cut-off was set as the median percentile, lower stocking density was associated with improvements in body condition score (BCS), coat cleanliness and bedding quantity, less coughing, less resting in a standing position, and less feeding related to the greater space available at the feed bunk. When the 75th percentile cut-off was used, indicators that improved were coat cleanliness, bedding quantity and mane and tail condition, as well as less resting in standing position and less feeding related to the greater space available at the feed bunk. Accordingly, the use of two different stocking density cut-off values showed that the increase of space allowance affected specific welfare indicators. Further increment of space and/or changes in management regimes should be investigated to improve all the indicators. Moreover, results related to feeding indicated the need to intervene as starch intakes exceeded recommended safe levels, negatively affecting horse welfare.

Keywords: welfare; horse; meat production; stocking density; feeding management

1. Introduction

Faostat data [1] indicate that more than half a million horses are slaughtered in Europe each year for meat production. In the past, most horse meat was derived from the slaughter of horses at the end of their working lives, whereas, nowadays, horse meat is mainly obtained from the specific breeding of heavy draft breeds [2]. According to Tateo et al. [3], farms breeding horses for meat primarily rear young horses. To increase their meat production performances, these farms apply intensive farming systems. However, concerns about animal welfare related to overcrowding and intensive feeding regimes have been raised over intensive farming systems [4]. High-density group housing can negatively affect horse welfare, influencing both their health and behaviour [5]. Moreover, in order to reduce the length of the fattening period and obtain fast increases in body weight, breeders often feed the animals with a high-starch diet. However, it is well known that feeding horses with high amounts of concentrates can negatively affect their intestinal health, increasing the risk for colic and gastrointestinal disorders [6].

Several studies have underlined the negative effects of high stocking density on the welfare of livestock species [7–9]. However, to the best of our knowledge, no research today has focused on horses farmed for meat production. Few studies have evaluated the effects of space allowance on the welfare of horses, and they are mainly based on some behavioural or physiological aspects [10–13]. However, stocking density is recognised as crucial to reaching an adequate level of welfare at farm level. [14]. The general approach of the European Union (EU) to ensure farm animal welfare is to increase the space allowance per animal [15]. Accordingly, the minimum space requirements in group housing systems have been set for pigs [16], poultry [17], and cattle [18]. However, no specific EU Directives are defined for meat production horses [19]. The first indications about minimum space requirements for horses housed in group pens have been provided by the Swiss Federal Council in the Animal Welfare Ordinance (TSchV) of 23 April 2008 [20]. In this document, the minimum space allowance per horse is based on the withers height of the individual group members. According to Burla et al. (2017) [11], these minimal requirements are not based on scientific evidence and may not be adequate to guarantee adequate welfare for all horses of a given group [11]. At the European Union level, this criterion was then adopted in the Animal Welfare Indicators (AWIN) welfare assessment protocol for horses [21].

The AWIN protocol is based on the assessment of animal-based indicators and follows the Welfare Quality® approach that consists of four welfare principles and twelve welfare criteria [22]. The four welfare principles are good feeding, good housing, good health, and appropriate behaviour. They represent the founding elements of the Five Freedoms [23] since they describe the needs of animals that should be satisfied in order to cover all aspects of animal welfare [22]. However, according to Mellor [24], affective outcomes are not sufficiently addressed in the Welfare Quality® system, being addressed only briefly in the list of welfare criteria. Indeed, the four welfare principles are primarily structured to evaluate specific physical/biological functions, so they have a predominantly physiological orientation. However, as Mellor discusses [24], it is necessary to identify how physical/biological imbalances can influence the affective state and, consequently, the welfare of the animals. For this reason, Mellor proposes the Five Domains model that includes the fifth "mental" domain, the aim of which is to evaluate the animals' mental state.

The study of animal welfare requires a multidimensional approach that involves the examination of a panel of welfare indicators encompassing all components of animal welfare [25]. Accordingly, welfare assessment involves three categories of indicators: resource-based, management-based, and animal-based [26,27]. Some criticisms have been made regarding the application of protocols built on animal-based indicators due to the difficulty in applying them at the farm level—the protocols being very time-consuming and costly [28]. Indeed, animal welfare is not an easy subject to study,

and identifying the best protocol to apply on any given farm is difficult. The European Commission has financed the development of a specific protocol that considers animal-based indicators to assess and promote horse welfare—the AWIN protocol—presently the only tool validated by the European Commission for the assessment of equine welfare.

However, when the aim is to assess equine welfare on farms geared towards meat production, some limitations of the AWIN protocol become evident. As clearly underlined in the section dedicated to horses housed in groups pens, the AWIN protocol still needs to be refined and improved in light of the results of up-to-date scientific research on horses reared in this manner. Moreover, the AWIN protocol was developed in relation to horses aged 5 years or older. As such, it is imperative that this tool is revised for its use on intensive breeding farms that rear young horses (less than 5 years old) in high-density group pens.

In the present study, a checklist adapted from the AWIN protocol and based on the Welfare Quality® principles was developed to evaluate whether the welfare indicators selected were influenced by the main causes of concern that regard intensive breeding farms: stocking density and feeding management. We hypothesise that welfare would be poorer at higher stocking densities and that some welfare indicators could be negatively affected by the feeding strategies adopted with meat production in mind. We tested the effect of two different stocking density cut-off values (the median and 75th percentile values), dividing the data into two groups (low vs. high stocking densities) to assess whether any improvements in horse welfare could be observed with even a small increase in space allowance per horse.

2. Materials and Methods

The present study was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy, Prot.n.2202, 8/04/2019). It was conducted in the presence of representatives of the Regional Veterinary Services. The owner of the horses agreed to the purpose of the study.

2.1. Data Collection

The welfare assessment was carried out on the biggest horse breeding farm for meat production in northern Italy. Seven surveys were conducted between April and June. The surveys commenced two hours after the morning meal and lasted approximately 3.5 h (from 9:00 a.m. until 12:30 p.m.).

The farm in question adopts intensive farming methods and, at any one time, houses around 300 young horses belonging to different breeds of both sexes—colts (not gelded) and fillies aged 16 ± 8 months (mean ± standard deviation (SD)). It sends a total of 2000 animals to slaughter each year. The horses were housed in group pens situated in a barn with two open sides; they had no access to any outdoor paddock area. Pens were enclosed by horizontal metal rail bars, which also delimited the pens at the feed bunk level. One automatic drinker providing tap water was available in each pen independent of the number of the animals enclosed. The floor was concrete and covered with barley straw bedding once a day before the evening meal by an automatic straw-dispersing tractor programmed to cover the entire pen floor with a thickness of at least 15 cm of straw. The number of animals per pen varied, and male and female horses were not separated. Horses were not fed on an individual basis; instead, twice a day (7:00 a.m. and 6:00 p.m.), each pen was provided with long stem self-produced meadow hay (approximately 6 kg/animal/day) and an amount of pelleted feed equal to 8 kg/animal/day. The pelleted feed was a cereal-based commercial feed (complementary feed; labelled to contain crude protein 14.50%, ether extract 3.50%, crude fibre 5.70%, ash 6.60%; as fed: starch 55%).

The farm contained a total of 24 pens; of these, every second pen was selected for assessment, providing a total of 12 pens for evaluation by means of the seven surveys conducted over the study timespan. At the time of each survey, the horses in each group pen had changed, as had the number of animals it contained. As such, different stocking densities could be evaluated by means of the welfare assessment checklist. Table 1 reports the physical characteristics of the 12 selected pens, and the median

and 25th–75th percentile values regarding the number and height of the horses housed within each pen for the seven surveys conducted.

Table 1. Area (m^2) and feed bunk length (m) of the 12 multiple pens evaluated in the seven surveys conducted between April and June. The median values (plus 25th–75th percentiles) for the number and the height (at the withers) of the horses within each pen are reported.

Pen ID	Area of the Pen (m^2)	Length of Feed Bunk (m)	Number of Horses Median (25th–75th)	Height at the Withers (cm) Median (25th–75th)
1	18.1	3.9	2.5 (2–3)	150 (145–150)
2	14.9	3.2	4 (4–4)	140 (137.5–140)
3	20.8	4.6	4 (4–5)	140 (140–140)
4	22.5	4.7	5 (5–6)	140 (140–143.8)
5	16.5	4.0	5 (4–5)	140 (136.3–147.5)
6	27.7	6.7	7 (7–7.75)	140 (130–140)
7	35.0	7.0	9.5 (9–10)	140 (140–150)
8	38.0	7.6	10 (9–11)	130 (130–132.5)
9	36.0	4.8	8 (7.5–8)	147.5 (141.3–153.8)
10	36.8	4.9	10 (9–11)	140 (136.3–140)
11	34.9	4.7	12 (10–13)	140 (140–145)
12	46.5	6.2	15 (14–15)	125 (125–125)

2.2. Welfare Assessment Checklist

A checklist adapted from the AWIN welfare assessment protocol for horses [21] was employed by five equine veterinarians who are experts on welfare protocols. Before starting the study, the evaluators received specific training on the welfare checklist, and at the end of the training period, interobserver reliability was evaluated, as indicated in the statistical analysis section.

Table 2 shows the welfare assessment checklist developed and used by the evaluators. Each evaluator independently filled out his/her own checklist. The checklist contained four sections, each regarding one of the four welfare principles of the Welfare Quality® approach: good feeding, good housing, good health, and appropriate behaviour. Horse welfare was assessed according to the welfare criteria and welfare indicators belonging to each principle. The welfare indicators included resource-based, management-based, or animal-based indicators and are written in bold font in the following sections.

Table 2. Welfare assessment checklist used in each of the seven surveys. The checklist is divided into four sections corresponding to the Welfare Quality® principles: good feeding, good housing, good health, and appropriate behaviour. Each principle is measured using specific resource-based, management-based, and animal-based indicators. Each section is accompanied by detailed guidance notes and photographs illustrating the scores.

Welfare Principles	Welfare Criteria	Welfare Indicators	Score	Notes
Good feeding	Appropriate nutrition	N of horses within the group pen	□ … …	
		BCS [1]	□ N of horses scored as Thin … □ N of horses scored as Normal … □ N of horses scored as Fat …	 Thin Normal Fat
		Length of the feed bunk	□ … … m	Consider as adequate space at the feed bunk of at least 1 m per horse (m/horse)
		Space allowance per horse at the feed bunk (m/horse) [2]	□ Adequate □ Inadequate	
	Absence of prolonged thirst	Water availability [3]	□ Adequate □ Inadequate	Consider the functioning of the automatic drinkers
		Water point cleanliness [3]	□ Clean: Bowl and water are clean □ Partly dirty: Bowl is dirty but water is clean □ Dirty: Bowl and water are dirty	 Clean Partly dirty Dirty
	Comfort around resting	Bedding quantity [4]	□ Adequate □ Inadequate	 **Adequate (100% of covered floor)** **Adequate (≥70% of covered floor)** **Inadequate >30% of not covered floor)**

Table 2. *Cont.*

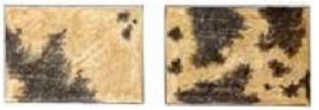
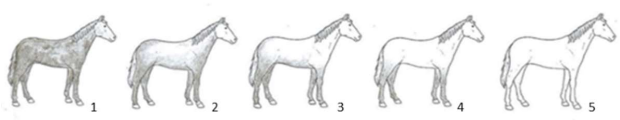
Welfare Principles	Welfare Criteria	Welfare Indicators	Score	Notes
Good housing		Bedding cleanliness [5]	□ Adequate □ Inadequate	 Adequate (≥70% of clean bedding)  Inadequate (>30% of dirty bedding)
		Coat cleanliness [6]	□ N of horses scoring 1 … □ N of horses scoring 2 … □ N of horses scoring 3 … □ N of horses scoring 4 … □ N of horses scoring 5 …	
	Thermal comfort	Environmental temperature (°C) [7]	□ Adequate □ Inadequate	Environmental temperature is considered adequate if it ranges between +5–+25 °C
		Environmental humidity (%) [7]	□ Adequate □ Inadequate	Environmental humidity is considered adequate if it ranges between 60–80%
	Ease of movement	Area of the pen (m^2)	□ ..... m^2	Medium height at the withers Available space per horse (m^2/horse)
		Medium height at the withers of the horses within the pen	□ … … cm	<120 cm 5.5 m^2
		Stocking density (m^2/horse) [8]	□ Adequate □ Inadequate	120–148 cm 7 m^2
				148–162 cm 8 m^2
				162–175 cm 9 m^2

Table 2. *Cont.*

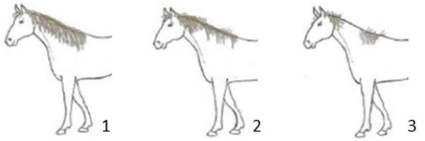
Welfare Principles	Welfare Criteria	Welfare Indicators	Score	Notes
Good health	Absence of injuries	Integument alterations [3]	N of horses within the pen that present integument alterations … … .	Consider integument alteration: area of alopecia, skin lesions as superficial would or deep wound, tumefaction, and swelling
		Mane condition [9]	□ N of horses with a mane score of 1 … □ N of horses with a mane score of 2 … □ N of horses with a mane score of 3 …	
		Tail condition [9]	□ N of horses with a tail score of 1 … □ N of horses with a tail score of 2 … □ N of horses with a tail score of 3 …	
		Swollen joints/signs of lameness [10]	N of horses within the pen that present swollen joints/signs of lameness … …	Focus attention on distal legs, the shape of the hoof and the animals' movements
	Absence of diseases	Coughing [10]	N of horses within the pen with coughing … …	Evaluate coughing together with breathing assessment
		Abnormal breathing [10]	N of horses within the pen with abnormal breathing … …	Consider breathing abnormal if the horse shows any of the following signs: flared nostrils, extended head and neck, increased respiratory rate, or asynchrony between movements of the chest and the abdomen
		Discharges [10]	N of horses within the pen with discharges … …	Consider nasal and ocular discharges
		Consistency of faeces [11]	□ Normal □ Abnormal	 Normal Abnormal

Table 2. *Cont.*

Welfare Principles	Welfare Criteria	Welfare Indicators	Score	Notes
	Absence of pain and pain induced by management procedures	State of the awareness	N of horses within the pen with an abnormal state of the awareness … … …	State of awareness is considered abnormal if horses appear apathetic, depressed, alarmed, in a state of stupor
Appropriate behaviour	Expression of social behaviour	Mutual grooming	N of horses within the pen … …	Body cleaning is performed by one horse towards a conspecific or reciprocally
		Playing	N of horses within the pen … …	Horse plays alone or with other horses. It includes playing with structural parts of the pen, locomotor play, play fighting
	Expression of other behaviours	Feeding	N of horses within the pen … …	Horse eats hay, straw or feedstuff in the trough or on the ground
		Watching	N of horses within the pen … …	Horse is in a standing position. The expression is attentive, observing the surroundings
		Resting in standing position	N of horses within the pen … …	Horse is in a standing position. The expression is relaxed
		Resting in lying position	N of horses within the pen … …	Horse is lying on the ground in sternal position with the limbs flexed below the body or in lateral position with extended limbs
		Sexual behaviours	N of horses within the pen … …	Stallion sniffs or bites the mare's genitals. The stallion mounts the mare
		Aggressive behaviours	N of horses within the pen … …	They include snaking (horse stretches its neck towards a conspecific with ears pinned back, threatening to bite); kicking (horse makes a kicking movement towards another horse with one or both hind limbs); biting (horse touches the body of another horse using its teeth whilst its ears are turned backwards).
		Stereotypic behaviours	N of horses within the pen … …	Horse presents stereotypic behaviour: oral and/or locomotor stereotypic behaviours

[1] BCS was scored as thin, normal, or fat on the basis of the visual appraisal of the shape of each animal. [2] Space allowance at the feed bunk was considered adequate if it allowed at least 1 m per horse, as per the suggestions provided by the Code of Practice for the Care and Handling of Equines [29]. [3] Scores adapted from Animal Welfare Indicators (AWIN) welfare assessment protocol for horses [21]. Water availability was assessed adequate when automatic drinkers were functioning. [4] A specific scoring system was developed by the authors to evaluate bedding quantity. Bedding quantity was scored as adequate if ≥70% of the floor was covered by bedding. Bedding quantity was scored as inadequate if >30% of the floor was not covered by bedding. [5] A specific scoring system was developed by the authors to evaluate bedding cleanliness. Bedding cleanliness was scored as adequate if ≥70% of the bedding was clean, and inadequate when >30% of the bedding was dirty. [6] Specific 5-point scoring system developed for the assessment of coat cleanliness: 1: coat completely dirty; 2: dirty limbs, abdomen, barrel, flanks, and neck; 3: dirty limbs, and abdomen; 4: dirty limbs; 5: coat completely clean. [7] Scores adapted from Wageningen UR Livestock Research Welfare Monitoring System Assessment protocol for horses [30]. Temperature was considered adequate when it was within the horse's thermoneutral zone (+5 to +25 °C). Relative humidity was deemed to be adequate when the values ranged from 60 to 80%. [8] Stocking density was considered adequate according to the indications reported in the associated guidance notes adapted from the AWIN protocol in the section for group-housed horses [21] (i.e., if horses are assessed to measure between 120 and 148 cm at the withers, a minimal space of 7 m^2/horse is required to be considered adequate). [9] Specific 3-point scoring system defining mane and tail condition: 1: mane/tail are in good condition for their entire length; 2: areas of broken and/or absent mane or tail hair, but intact skin; 3: areas of broken and/or absent mane or tail hair and damaged skin. [10] Scores adapted from AWIN welfare assessment protocol for horses [21]. [11] Faeces were scored as normal if the shape of the faeces was conserved.

2.2.1. Good Feeding

The welfare principle "good feeding" was described by its two welfare criteria: "appropriate nutrition" and "absence of prolonged thirst".

To assess "appropriate nutrition", the body condition score (BCS) was rated and recorded. The BCS is the only welfare indicator used in the AWIN protocol to describe the welfare criteria "appropriate nutrition". It is scored using a 5-point scale [31] in which the nutritional status of an animal is assessed through observation and palpation of anatomical key areas. In the present study, the BCS of the horses was scored as "thin", "normal" or "fat" by means of the visual appraisal of the animals' shape alone, since it was not possible to touch the horses during the assessment (see Table 2, with associated guidance notes and illustrative photographs). The number of horses per pen judged as "thin" was recorded and used in the statistical analysis. This study also considered space allowance at the feed bunk as a welfare indicator of "appropriate nutrition" since easy access to feed troughs must be guaranteed to ensure the welfare of animals in production systems [32]. Space allowance at the feed bunk (m/horse) was calculated by dividing the length of the feed bunk (meters) by the number of horses within the pen.

The welfare criterion "absence of prolonged thirst" was assessed by considering water availability and water point cleanliness. Water availability was assessed by evaluating the correct functioning of the automatic drinkers. Water point cleanliness was scored as suggested by the AWIN protocol; specifically, the drinkers were scored "dirty" if both the bowl and water were dirty (i.e., the presence of organic materials, such as feed, soil or faeces); "partly dirty" if the bowl was dirty but the water clean, or "clean" if both bowl and water were clean (see Table 2 with associated guidance notes and illustrative photographs). The frequency (%) of the automatic drinkers scored as adequate or inadequate was calculated and used in the statistical analysis.

2.2.2. Good Housing

The welfare principle "good housing" includes the welfare criteria "comfort around resting", "thermal comfort" and "ease of movement".

Comfort around resting was evaluated by considering the two welfare resource-based indicators, "bedding quantity" and "bedding cleanliness", as used in the AWIN protocol, plus "coat cleanliness".

The AWIN protocol scores the former two indicators in a qualitative manner only through the use of pictures. Here, in order to achieve a more standardised method, we developed a specific scoring system to evaluate bedding quantity and cleanliness. Bedding quantity was scored as adequate when ≥70% of the floor was covered (defined in the AWIN protocol as "sufficient bedding material"), and inadequate if >30% of the floor was not covered (defined in the AWIN protocol as "no bedding material" and "insufficient bedding material"; see Table 2 with its detailed guidance notes and photographs illustrating the scores). Bedding cleanliness was scored as adequate if ≥70% of the bedding was clean (defined in the AWIN protocol as "clean bedding material") and inadequate when >30% of the bedding was dirty (defined in the AWIN protocol as "dirty bedding material"; see Table 2 with its detailed guidance notes and photographs illustrating the scores). For the statistical analysis, bedding quantity and bedding cleanliness were expressed as frequencies (%) of scores.

Coat cleanliness was also taken into consideration for the assessment of "comfort around resting". We decided to evaluate this welfare indicator as it reflects the environmental conditions in which the animals are kept. A specific 5-point scoring system was designed to assess coat cleanliness (see Table 2 with its detailed guidance notes and photographs illustrating the scores). Horses were assigned a score of 1 if they were completely dirty; a score of 2 if they presented dirty limbs, abdomen, barrel, flanks and neck; a score of 3 for dirty limbs, and abdomen; a score of 4 for dirty limbs only; a score of 5 for a completely clean horse. A coat cleanliness score of 1, 2 or 3 was rated "dirty". The number of horses per pen rated as dirty was used for the subsequent statistical analysis.

For the welfare criterion "thermal comfort", since it was not possible to evaluate this parameter by examining whether the animals that showed clinical signs of thermal stress, as suggested in the

AWIN protocol, thermal comfort was instead evaluated through the measurement of environment temperature (°C) and relative humidity (%). These measurements were taken in front of each pen using a digital thermometer and hygrometer. According to the Wageningen UR Livestock Research Welfare Monitoring System [30], the temperature was considered adequate when it was within the horse's thermoneutral zone (+5 °C to +25 °C); and relative humidity was deemed to be adequate when the values ranged from 60% to 80%.

The welfare criterion "ease of movement" should regard the quality of the exercise horses are able to partake in. The AWIN protocol describes this management-based indicator by referring to the possibility for animals to spend part of their day performing activities in outdoor areas. Since it was not possible to apply this welfare indicator in the evaluation of animals kept in a production system, we decided to evaluate each pen's area (m^2) and stocking density (m^2/horse) to gain some data pertaining to the animals' possibility for "ease of movement". Once the area of a pen was calculated, it was then divided by the median height of the horses, measured to the withers, within the pen. As we were not able to touch the animals, a laser meter was used to measure the height of animals at the withers. Measurements were conducted for the tallest and the shortest horse in order to ascertain the height range for the horses within a pen. The measurement was made at the moment in which the animal was standing in a position that was parallel to the wall or to the horizontal metal rail bars. The stocking density was considered adequate or inadequate according to the indications provided in the AWIN protocol in the section adapted for group-housed horses [21]. Accordingly, if animals were assessed to measure between 120 cm and 148 cm at the withers, a minimal space of 7 m^2/horse was required, whereas if the heights ranged between 148 cm and 162 cm, an adequate space allowance should not be less than 8 m^2/horse.

2.2.3. Good Health

The welfare principle "good health" includes three welfare criteria: "absence of injuries", "absence of diseases", and "absence of pain and pain induced by management procedures".

"Absence of injuries" is described by evaluating the animal-based indicators "presence of integument alterations" and "presence of swollen joints—signs of lameness", as well as "mane condition" and "tail condition".

The presence of integument alterations was evaluated by recording the extent of visible areas of alopecia, skin lesions (as superficial or deep wounds), tumefaction, and swelling. Since it was not possible to approach the animals, a visual inspection of the body of each animal was performed. In the checklist, the number of horses within each pen presenting at least one visible integument alteration was recorded and used for statistical analysis.

The number of horses presenting visibly swollen joints and/or signs of lameness was recorded. In addition, a visual inspection of the body of each horse within the pen was performed, focusing attention on the distal limbs, the shape of the hooves, and the animals' movements.

In our assessment of the welfare criterion "absence of injuries", we decided to introduce two additional animal-based indicators on the basis of their initial observations of the animals; they were mane condition and tail condition. We decided to include these welfare indicators as they seemed to reflect the specific housing and management features of this kind of farm. In particular, the observation of alterations to the mane and/or tail seemed to constitute a specific "occupational ailment" in this specific context. A specific 3-point scoring system was defined for both mane and tail condition: a score of 1 indicated good mane/tail condition for their entire length; a score of 2 indicated areas of broken and/or absent mane/tail hair, but with the skin intact; and a score of 3 indicated a damaged mane/tail with areas of broken and/or absent mane or tail hair and injured skin (see Table 2 with its detailed guidance notes and the photographs illustrating the scores).

The welfare criterion "absence of diseases" was assessed using four animal-based welfare indicators: "coughing", "abnormal breathing", "discharges", and "consistency of faeces". Coughing and abnormal breathing were recorded as the number of horses presenting these symptoms. To evaluate

breathing, the head and the flanks of each horse were observed. Breathing was considered abnormal when at least one of the following clinical signs were observed: flaring of the nostrils, extended head and neck, increased respiratory rate, or asynchrony between movements of the chest and the abdomen. The number of horses within the group pen coughing or with abnormal breathing was recorded and used in the statistical analysis. Nasal and ocular discharges were evaluated by observation. This assessment was performed at the same time as the assessment for coughing and abnormal breathing. Once again, the number of horses within the group pen presenting these clinical signs was recorded.

The consistency of faeces was considered by evaluating the shape of the faeces present in the bedding of each group pen and recorded as normal and/or abnormal. Faeces were scored as abnormal if the shape of the faeces was not conserved. For statistical analysis, the frequency (%) of group pens containing abnormal faeces was calculated.

To assess the welfare criterion "absence of pain and pain induced by management procedures", the indicator "state of awareness" was evaluated. The AWIN protocol recommends the use of the Horse Grimace Scale that assesses equine facial expressions for the assessment of pain; however, this was not deemed feasible in the present study, thus the concept of state of awareness was introduced as an alternative. This involved observing the animals and noting whether they presented any symptoms of an "abnormal" state of awareness, which includes the adoption of a depressed or an alarmed stance, paying no attention to the surrounding environment and an inadequate response to stimuli, such as light, noise and the presence of people. The number of horses per pen that presented an abnormal state of awareness was recorded and used in the statistical analysis.

2.2.4. Appropriate Behaviour

To assess the welfare principle "appropriate behaviour", the following welfare indicators were considered (as measures of the welfare criteria "expressions of social behaviour" and "expressions of other behaviours"): feeding, watching, mutual grooming, resting in a standing position, resting in a lying position, playing, sexual behaviours, aggressive behaviours, and stereotypic behaviours (licking, crib-biting, weaving, head nodding, wood chewing; see Table 2 with its detailed guidance notes). To assess these indicators, all five evaluators simultaneously observed the horses within a single pen. They were positioned at different positions outside the pen at a maximum distance of 5 m from the horses. The welfare assessment started 5 min after the placement of the evaluators, who remained still and quiet to allow the horses to become accustomed to their presence. A methodology was adapted that involved observing the horse situated the furthest to the left in the pen, then moving to the animal situated to its right, and so on. The number of horses displaying each specific behaviour was recorded and used for statistical analysis.

2.3. Statistical Analysis

For analytical purposes, the data pertaining to the individual group pens were assigned to one of two groups on the basis of their stocking density (m^2/horse). The median stocking density was calculated in order to divide the data into two groups, depending on whether they were housed at a low stocking density (LSD^{50th}; i.e., at or above the 50th percentile) or a high stocking density (HSD^{50th}; below the 50th percentile). The 75th percentile value was also calculated, and the animals again divided into low or high stocking density groups depending on whether they were housed at or above, or below the 75th percentile stocking density (LSD^{75th} and HSD^{75th}, respectively).

Data were analysed using IBM SPSS® Statistics 21.0 software (SPSS Inc., Chicago, IL, USA) to identify any differences between the groups divided according to the stocking density cut-off values. The Shapiro–Wilk test was used to assess whether the data were distributed according to a normal distribution. Since the data were not normally distributed, the Mann–Whitney U and the Fisher's exact tests were applied. A p-value < 0.05 was considered significant to infer that differences between the groups were related to the stocking density.

The interobserver reliability of the expert evaluators in their assessment of welfare indicators was evaluated by means of the Cohen's kappa coefficient (K).

Dichotomous variables (bedding cleanliness, bedding quantity, consistency of faeces, water point cleanliness) were expressed as frequencies (% of group pens). The other welfare indicators (i.e., the nondichotomous variables) were expressed as the number (N) of horses within each group pen presenting a specific score or health condition or performing a specific behaviour.

3. Results

A total of 561 horses were evaluated. The horses belonged to Italian or French heavy draft breeds, and the mean age (± SD) was 16 (± 8) months.

The median values (plus 25th–75th percentiles) for environment temperature (°C) and relative humidity (%) over the seven surveys were 13 °C (11–23 °C) and 73% (55–75%), respectively.

The Cohen's kappa coefficients (Ks) for interobserver reliability ranged between 0.61 and 1, indicating substantial (K = 0.61–0.80) to strong (K = 0.80–1) agreement between the expert evaluators.

3.1. Results Considering the Median Cut-Off Value for the Stocking Density

Table 3 shows the results of the Mann–Whitney U-test and Fisher's exact tests. The median cut-off value for the stocking density was calculated in order to divide and compare the survey data according to whether the horses were housed at a low stocking density (LSD^{50th}) or a high stocking density (HSD^{50th}). The median cut-off value for the stocking density (m^2/horse) was 3.95 m^2/horse (LSD^{50th} group ≥3.95 m^2/horse vs. HSD^{50th} group <3.95 m^2/horse).

When the two groups were compared on the basis of the median stocking density cut-off value, significant differences were found in two of the welfare indicators of good feeding: the space at the feed bunk (m/horse; $p < 0.001$) and the BCS ($p = 0.004$). The ideal feeding space per horses at a feed trough is reported to be 1 m/horse [29]; the median space (plus 25th–75th percentiles) revealed here was 0.95 (0.70–1.30) m/horse for the LSD^{50th} group and 0.6 (0.42–0.79) m/horse for the HSD^{50th} group. Moreover, the median number of horses within the group pens scored as thin was higher for the horses in the HSD^{50th} group at 0.5 (0–2.25) compared with 0 (0–0) for the LSD^{50th} group.

Considering the welfare principle of good housing, the welfare indicators "coat cleanliness" and "bedding quantity" were shown to be influenced by the stocking density. The median number of animals scored as having a dirty coat (coat cleanliness score of 1 to 3) was lower (3, 1–4) in the LSD^{50th} group than in the HSD^{50th} group (5, 2–7) ($p = 0.004$). Therefore, a higher stocking density was associated with a significantly higher number of horses scored as having a dirty coat. The frequency (%) of pens scored as having an inadequate quantity of bedding was 56.8% in the LSD^{50th} group and 83.3% in the HSD^{50th} group ($p = 0.021$), revealing that when horses were housed at higher densities, a significantly higher percentage of pens had inadequate amounts of bedding material covering the pen floor.

For the welfare principle of good health, just one welfare indicator was affected by stocking density: the median number of horses with a cough was significantly lower in the LSD^{50th} group than in the HSD^{50th} group ($p = 0.028$).

Finally, with regard to the welfare principle of appropriate behaviour, two indicators were affected by stocking density: feeding behaviour and resting in a standing position. The median number of horses exhibiting feeding behaviour at the moment of the observation was significantly higher in the HSD^{50th} group (5, 2–6.75) than the LSD^{50th} group (2, 0.5–4) ($p = 0.001$). This suggests that, on the farm in question, horses housed at a higher stocking density are more likely to express feeding behaviour. Moreover, with regard to resting in standing position, more animals were found to express this behavior in the HSD^{50th} group (1, 0–3) than LSD^{50th} (0, 0–2) ($p = 0.012$).

Table 3. Statistical analysis performed using the median cut-off value for the stocking density (3.95 m^2/horse). Nondichotomous variables are expressed as the median number of horses (plus 25th–75th percentiles) within pens that show a specific score or health condition or are performing a specific behaviour. Space at the feed bunk is expressed as the median (plus 25th–75th percentiles) length in metres available per horse. Nondichotomous variables were analysed using the Mann–Whitney U test: the test statistic (U) and *p*-values are reported. Dichotomous variables are expressed as frequencies (%) and were analysed using the Fisher exact test: the test statistic (χ^2) and *p*-values are reported. Data were considered significant for *p*-values < 0.05.

Welfare Principle	Welfare Indicator	LSD^{50th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (*n* = 37) with ≥3.95 m^2/Horse	HSD^{50th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (*n* = 36) with <3.95 m^2/Horse	Test Statistics § Mann–Whitney U Test (U) Fisher Exact Test (χ^2)	*p*-Values
Good feeding	Space at feed bunk (m/horse)	0.95 (0.70–1.30)	0.6 (0.42–0.79)	U = 194.00	<0.001 *
	BCS [0]	0 (0–0)	0.5 (0–2.25)	U = 459.00	0.004 *
	Water point cleanliness [a]	Adequate: 68.6% Inadequate: 31.4%	Adequate: 63.9% Inadequate: 36.1%	χ^2 = 0.174	0.803
Good housing	Coat cleanliness [1]	3 (1–4)	5 (2–7)	U = 408.50	0.004 *
	Bedding cleanliness [a]	Adequate: 22.9% Inadequate: 77.1%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 0.387	0.757
	Bedding quantity [a]	Adequate: 43.2% Inadequate: 56.8%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 6.121	0.021 *
Good health	Skin lesions [2]	1 (0.5–2)	1 (0–2)	U = 658.50	0.931
	Mane condition [3]	4 (3–7)	5.5 (3–9.5)	U = 389.00	0.142
	Tail condition [4]	1 (0–1.5)	1.5 (0–4)	U = 470.00	0.056
	Swollen joints [5]	0 (0–1)	1 (0–2)	U = 602.00	0.444
	State of awareness [6]	0 (0–0)	0 (0–0)	U = 610.50	0.075
	Abnormal breathing [7]	1 (0–1)	0 (0–0.75)	U = 631.50	0.626
	Nasal discharges [8]	0 (0–2)	0 (0–1)	U = 574.00	0.249
	Ocular discharges [9]	0 (0–1)	0 (0–1)	U = 650.00	0.833
	Consistency of faeces [a]	Adequate: 0% Inadequate: 100%	Adequate: 8.3% Inadequate: 91.7%	χ^2 = 3.215	0.115
	Cough [a]	0 (0–0)	0 (0–1)	U = 522.00	0.028 *

Table 3. *Cont.*

Welfare Principle	Welfare Indicator	LSD[50th] Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 37) with ≥3.95 m^2/Horse	HSD[50th] Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 36) with <3.95 m^2/Horse	Test Statistics [§] Mann–Whitney U Test (U) Fisher Exact Test (χ^2)	p-Values
Appropriate behaviour	Feeding [10]	2 (0.5–4)	5 (2–6.75)	U = 353.50	0.001 *
	Watching [11]	1 (0–3)	1.5 (0–4)	U = 598.00	0.442
	Mutual grooming [12]	0 (0–0)	0 (0–0)	U = 648.50	0.574
	Resting in a standing position [13]	0 (0–2)	1 (0–3)	U = 452.00	0.012 *
	Resting in a lying position [14]	0 (0–0)	0 (0–1)	U = 597.50	0.306
	Playing [15]	0 (0–0)	0 (0–0)	U = 623.00	0.574
	Sexual behavior [16]	0 (0–0)	0 (0–0)	U = 646.50	0.532
	Aggressive behavior [17]	0 (0–0)	0 (0–0)	U = 566.00	0.076
	Stereotypic behavior [18]	0 (0–0)	0 (0–0)	U = 666.00	1

* Significant values. [§] The degrees of freedom for each analysed variable were equal to 1. [a] Dichotomous variables expressed as frequencies (%) of occurrence within the multiple pens. [0] N of horse scored as thin using the specifically developed 3-point scoring system. [1] N of horses with a coat cleanliness score of 1, 2 or 3, using the specifically developed 5-point scoring system. [2] N of horses within the pens presenting skin lesions, including areas of alopecia, injuries, tumefaction, or swelling. [3] N of horses presenting a ruined mane, as defined by a score of 3, using the specifically developed 3-point scoring system. [4] N of horses presenting a ruined tail, as defined by a score of 3, using the specifically developed 3-point scoring system. [5] N of horses presenting swollen joints. [6] N of horses presenting an abnormal state of awareness. [7] N of horses presenting abnormal breathing. [8] N of horses presenting nasal discharges. [9] N of horses presenting ocular discharges. [10] N of horses feeding. [11] N of horses watching. [12] N of horses engaged in mutual grooming. [13] N of horses resting in a standing position. [14] N of horses resting in a lying position. [15] N of horses playing. [16] N of horses performing sexual behaviours. [17] N of horses engaged in aggressive behaviours. [18] N of horses performing stereotypic behaviours.

3.2. Results Considering the 75th Percentile Cut-Off Value for the Stocking Density

The data were reanalysed by considering a stocking density (m^2/horse) cut-off value equal to the 75th percentile: 4.75 m^2/horse. This analysis was performed to assess whether a small increase in space allowance per horse would lead to any improvements in horse welfare. Therefore, animals in the LSD^{75th} group had a space allowance ≥4.75 m^2/horse, whereas those in the HSD^{75th} group had <4.75 m^2/horse. The results of the Mann–Whitney U test and the Fisher exact tests are shown in Table 4.

Considering the welfare principle of good feeding, once again, a significant difference was shown in relation to space at the feed bunk (m/horse; $p < 0.001$). When we consider a lower stocking density, this automatically correlates with a larger feeding space per animal at the feed bunk. In fact, the median space at the feed bunk was 1.3 (1.10–1.54) m/horse in the LSD^{75th} group vs. 0.70 (0.45–0.84) m/horse for the HSD^{75th} group.

Moving on to the welfare principle of good housing, the data regarding coat cleanliness and the bedding quantity were again found to differ significantly between the low and high stocking density groups. The median number of animals scored to have a dirty coat (cleanliness score of 1 to 3) was higher (4, 2–7) in the HSD^{75th} group than in the LSD^{75th} group (2, 1–4) ($p = 0.005$). The frequency (%) of pens scored as having an inadequate quantity of bedding was significantly lower (44.48%) in the LSD^{75th} group compared with the HSD^{75th} group (78.2%) ($p = 0.016$).

For the welfare principle of good health, both mane condition and tail condition were significantly influenced by stocking density when defining the groups by the 75th percentile cut-off, with *p*-values of 0.038 and 0.024, respectively. The median number of horses presenting a ruined mane (score of 3) was significantly higher (5, 3–8) in the HSD^{75th} group than the LSD^{75th} group (3.5, 3–4.75) ($p = 0.038$). Moreover, the median number of horses presenting a ruined tail (score of 3) was lower (0, 0–1) in the LSD^{75th} group than in the HSD^{75th} group (1, 0–3) ($p = 0.024$).

Considering the welfare principle of appropriate behaviour, the median number of horses expressing feeding behaviour at the moment of the welfare assessment was higher in the HSD^{75th} group (3, 2–6) than in the LSD^{75th} group (1.5, 0–3.25) ($p = 0.002$). A significant difference between groups was also found for the median number of horses standing in a resting position, which was higher in the HSD^{75th} group (1, 0–3) than the LSD^{75th} group (0, 0–0.25) ($p = 0.003$).

Interestingly, in contrast with the previous statistical analysis in which the median stocking density was used as the cut-off value, no statistical significance was shown for BCS or the presence of a cough when groups were compared on the basis of the 75th percentile cut-off value.

Table 4. Statistical analysis performed using the 75th percentile cut-off value (4.75 m^2/horse). Nondichotomous variables are expressed as the median number of horses (plus 25th–75th percentiles) presenting a specific score or health condition or performing a specific behaviour. Space at the feed bunk is expressed as median (plus 25th–75th percentiles) length in metres available per horse. Nondichotomous variables were analysed using the Mann–Whitney U test: test statistic (U) and *p*-values are reported. Dichotomous variables are expressed as frequencies (%) and were analysed using the Fisher exact test: the test statistic (χ^2), degrees of freedom and *p*-values are reported. Data were considered significant for *p*-values < 0.05.

Welfare Principle	Welfare Indicator	LSD^{75th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 18) with ≥4.75 m^2/Horse	HSD^{75th} Median Values (25th–75th Percentiles) and Frequencies (%) for groups (n = 55) with <4.75 m^2/Horse	Test Statistics § Mann–Whitney U test (U) Fisher Exact Test (χ^2)	*p*-Values
Good feeding	Space at feed bunk (m/horse)	1.3 (1.10–1.54)	0.70 (0.45–0.84)	U = 95.00	<0.001 *
	BCS [0]	0 (0–0)	0 (0–2)	U = 388.00	0.105
	Water point cleanliness [a]	Clean (0): 62.5% Dirty (1): 37.5%	Clean (0): 67.3% Dirty (1): 32.7%	χ^2 = 0.126 (1)	0.769
Good housing	Coat cleanliness [1]	2 (1–4)	4 (2–7)	U = 275.50	0.005 *
	Bedding cleanliness [a]	Adequate: 29.4% Inadequate: 70.61%	Adequate: 16.7% Inadequate: 83.3%	χ^2 = 1.275 (1)	0.299
	Bedding quantity [a]	Adequate: 55.6% Inadequate: 44.4%	Adequate: 21.8% Inadequate: 78.2%	χ^2 = 7.331 (1)	0.016 *
Good health	Skin lesions [2]	1 (0–2)	1 (0–2)	U = 443.50	0.49
	Mane condition [3]	3.5 (3–4.75)	5 (3–8)	U = 245.50	0.038 *
	Tail condition [4]	0 (0–1)	1 (0–3)	U = 313.50	0.024 *
	Swollen joints [5]	0 (0–1)	1 (0–2)	U = 374.50	0.095
	State of awareness [6]	0 (0–0)	0 (0–0)	U = 468.00	0.315
	Abnormal breathing [7]	0 (0–1)	0 (0–1)	U = 494.00	0.095
	Nasal discharges [8]	0 (0–2)	0 (0–1)	U = 484.00	0.873
	Ocular discharges [9]	0 (0–0)	0 (0–1)	U = 391.00	0.113
	Consistency of faeces [a]	Adequate: 0% Inadequate: 100%	Adequate: 5.5% Inadequate: 94.5%	χ^2 = 1.024 (1)	0.570
	Cough [a]	0 (0–0)	0 (0–0.5)	U = 420.00	0.183

Table 4. *Cont.*

Welfare Principle	Welfare Indicator	LSD^{75th} Median Values (25th–75th Percentiles) and Frequencies (%) for Groups (n = 18) with ≥4.75 m^2/Horse	HSD^{75th} Median Values (25th–75th Percentiles) and Frequencies (%) for groups (n = 55) with <4.75 m^2/Horse	Test Statistics [§] Mann–Whitney U test (U) Fisher Exact Test (χ^2)	p-Values
Appropriate behaviour	Feeding [10]	1.5 (0–3.25)	3 (2–6)	U = 260.50	0.002 *
	Watching [11]	1.5 (0–2.25)	1 (0–4)	U = 413.00	0.282
	Mutual grooming [12]	0 (0–0)	0 (0–0)	U = 449.00	0.087
	Resting in a standing position [13]	0 (0–0.25)	1 (0–3)	U = 277.50	0.003 *
	Resting in a lying position [14]	0 (0–0.25)	0 (0–0)	U = 489.00	0.917
	Playing [15]	0 (0–0)	0 (0–0)	U = 418.00	0.125
	Sexual behavior [16]	0 (0–0)	0 (0–0)	U = 468.00	0.315
	Aggressive behavior [17]	0 (0–0)	0 (0–0)	U = 396.00	0.120
	Stereotypic behavior [18]	0 (0–0)	0 (0–0)	U = 495.00	1

* Significant values. [§] The degrees of freedoms for each analysed variable were equal to 1. [a] Dichotomous variables expressed as frequencies (%) of occurrence within the multiple pens. [0] N of horses scored as thin using the specifically developed 3-point scoring system. [1] N of horses with a coat cleanliness score of 1, 2 or 3, using the specifically developed 5-point scoring system. [2] N of horses within the pens presenting skin lesions, including areas of alopecia, injuries, tumefaction, or swelling. [3] N of horses presenting a ruined mane, as defined by a score of 3, using the specifically developed 3-point scoring system. [4] N of horses presenting a ruined tail, as defined by a score of 3, using the specifically developed 3-point scoring system. [5] N of horses presenting swollen joints. [6] N of horses presenting an abnormal state of awareness. [7] N of horses presenting abnormal breathing. [8] N of horses presenting nasal discharges. [9] N of horses presenting ocular discharges. [10] N of horses feeding. [11] N of horses watching. [12] N of horses engaged in mutual grooming. [13] N of horses resting in a standing position. [14] N of horses resting in a lying position. [15] N of horses playing. [16] N of horses performing sexual behaviours. [17] N of horses engaged in aggressive behaviours. [18] N of horses performing stereotypic behaviours.

4. Discussion

The present study provides some information about the welfare status of horses reared for meat production and identifies some problems regarding this kind of intensive breeding system. The aim of the study was to test the hypothesis that stocking density and feeding management affect welfare indicators in horses reared for meat production. We decided to apply two different cut-off values when dividing the data according to stocking density to evaluate whether any improvements in horse welfare could be observed with even a small increase in space allowance per horse.

The assessment of animal welfare is a multidimensional and complex procedure that should include a combination of resource-, management- and animal-based indicators to describe the various aspects of animal welfare [33,34]. In the present study, we applied a welfare assessment checklist based on the AWIN structure. The AWIN protocol was specifically proposed and financed by the European Commission as an equine welfare assessment tool. However, as reported by AWIN, the protocol was developed for adult horses and may be difficult to apply to horses housed in group pens. As suggested by AWIN, the AWIN protocol needs to be redefined in light of up-to-date scientific research on horses kept in group pens and the specific breeding system being applied. To date, no studies have been published on the welfare of horses specifically bred on farms for meat production. As reported in the results section, the horses in the farming system studied were very young (16 ± 8 months, ±SD) and housed in groups, making it difficult to evaluate certain welfare indicators. In the present study, the key structure of the AWIN protocol was used, but certain adaptations were made to take into consideration the specific conditions of horse farming applied in this context (high stocking densities and an intensive feeding management regime).

Stocking density and adequate space allowance for horses housed in groups constitute one of the main welfare concerns regarding intensive horse farming for meat production [5]. The opportunity for movement is known to play an important role in equine welfare, having a positive effect on both physical and mental health [35]. Therefore, increasing the space allowance per horse is likely to form an important measure able to improve welfare. In the present study, the stocking density of group pens was calculated as m^2 available per horse (m^2/horse). Two stocking density cut-off values were calculated and considered in the statistical analyses, calculated as the median (3.95 m^2/horse) and the 75th percentile (4.75 m^2/horse) values. Recommendations relating to the minimum space needed per horse housed in groups are provided by the AWIN protocol that takes into consideration a horse's height, as measured at the withers. In the present study, the height of the horses within the group pens ranged between 120 and 160 cm. According to AWIN, horses in this height range require at least 7 m^2/horse. None of the pens at the farm provided this amount of space per horse. The use of two different stocking density cut-off values enabled us to show that an increase of just 0.80 m^2/horse (3.95 to 4.75 m^2/horse) was able to have a significant effect on specific welfare indicators.

Considering the welfare principle of good feeding, we revealed a significant influence of the space at the feed bunk (m/horse) at both cut-off values. The median feeding space per horse at the feed bunk was always less than 1 m/horse—the minimal distance recommended in the Code of Practice for the Care and Handling of Equines [29]—when the stocking density cut-off value was set to 3.95 m^2/horse. Adequate feeding space per horse at the feed bunk is important in order to mimic physiological feed intake behaviour and limit competition for resources [5,32]. Under natural conditions, horses live in herds and generally forage at the same time [36], preferring to maintain a distance of at least 2 m from each other [37]. In the HSD^{50th} condition of the present study, where the median space at the feed bunk was just 0.6 m/horse, the number of horses scored as "thin" according to the BCS scoring system was significantly higher than in the LSD^{50th} condition, where the feed bunk space was closer to 1 m/horse (0.95 m/horse). This difference was no longer present when the median feed bunk space of both groups exceeded 1 m/horse (i.e., when the 75th percentile cut-off was applied). It is interesting to notice that the number of animals exhibiting feeding behaviour was always greater in the high stocking density groups, independent of which cut-off value was used (HSD^{50th} or HSD^{75th}). This shows that both feed bunk space and stocking density can reciprocally influence feeding behavior.

For the welfare principle of good housing, we found stocking density to have a significant influence upon coat cleanliness. The number of animals rated as having a dirty coat was consistently higher in the high stocking density groups (HSD^{50th} and HSD^{75th}) compared with those housed at a lower stocking density (LSD^{50th} and LSD^{75th}). The welfare indicator "bedding quantity" was also judged as inadequate in both HSD^{50th} and HSD^{75th}. Indeed, when the lower cut-off value was set to 3.95 m^2/horse (i.e., the 50th percentile), the frequency of pens in the HSD^{50th} group scored as containing an inadequate quantity of bedding was 83.3%, whereas when the cut-off value was set to 4.75 m^2/horse (i.e., the 75th percentile), the frequency of pens scored as having an inadequate quantity of bedding was 78.2%. According to these results, when more animals are housed together, the frequency of inadequate quantities of bedding and the frequency of animals with dirty coats are higher. Indeed, the frequency of pens judged as having an inadequate level of bedding cleanliness exceeded 70% at all the stocking densities tested. This result may be a consequence of the high frequency of pens (>90%) containing abnormal faeces. The high prevalence of diarrhoea on the farm is probably related to the high level of starch in their diet. When the level of dietary starch exceeds the digestive capacity of a horse's small intestine, undigested starch may reach the hindgut where it undergoes rapid fermentation. The changes that may occur in the hindgut environment as a consequence of this starch, such as a decrease in luminal pH and marked changes in the microbial population, may lead to diarrhoea and an increased risk of colic [38,39].

Taken together, the results show that the amount and cleanliness of the pens' bedding were insufficient to provide an adequate level of environmental hygienic quality, which, in turn, influenced the cleanliness of the horses' coats. Moreover, we might hypothesise that horses housed at a higher density were more likely to consume the straw bedding to satisfy their natural need for foraging, especially at times when hay was not available [40], and this may have exacerbated the problem.

With regard to the welfare principle of good health, a number of welfare indicators were influenced by the stocking density. The number of horses presenting clinical signs of a cough was significantly higher in the HSD^{50th} group (<3.95 m^2/horse) compared with LSD^{50th} (≥3.95 m^2/horse). It is well known that increasing the stocking density increases the risk for transmission of respiratory diseases in stabled horses [41]. Thus, an increase in the per horse space allowance of a pen would be expected to decrease the occurrence of this indicator. Indeed, the statistical significance disappeared when the 75th percentile cut-off was applied.

No significant differences between groups were noted for either tail condition or mane condition when the stocking density cut-off value was set to 3.95 m^2/horse (50th percentile) since the scores of both groups revealed a high prevalence of mane and tail damage. However, differences were identified when the higher cut-off was applied, with horses allocated ≥4.75 m^2/horse (LSD^{75th}) less likely to incur mane or tail damage. A damaged tail might also be related to a major parasitic infestation that could cause excessive pruritus and lead to rubbing-induced injuries. It should be emphasized that no parasite management program was in force on this farm. Moreover, higher stocking densities may correlate with greater levels of contact made with the metal rail bars. It should also be noted that horizontal metal rail bars were in place at the feed bunks; thus, the crest of the neck was obliged to come into close contact with the metal rail bars during feed intake. Differences between groups were noted in the feeding behaviour of animals, independent of the cut-off value applied. However, the mane and tail condition differences were only noted when the higher cut-off value was applied, indicating that when animals were kept at stocking densities lower than 4.75 m^2/horse (HSD^{75th}), a higher number of animals incurred tail and mane damage, also due to the constraint of the metal rail bars at the feed bunks.

In addition to providing key information about health-related parameters, the direct observation of the animals is fundamental for gathering data on horse behaviour. This data is fundamental as it provides insight into how an animal perceives and interacts with its environment [42,43]. The affective state, as intended by Mellor in the fifth "mental" domain [24], is not considered by AWIN. The AWIN protocol assesses the emotional state through the evaluation of behaviours indirectly linked to positive

emotional states only, since appropriate behaviour represents the freedom of animals to express normal behaviour–intending behaviours that are as close as possible to those performed in nature [23]. Within the welfare principle of appropriate behaviour, we observed that a higher number of animals were feeding in the more densely housed groups for both cut-off values (HSD^{50th} and HSD^{75th}). However, feeding behaviour was spot sampled and may not indicate a long-term behaviour pattern. In contrast, the BCS constitutes a more direct indicator of feed intake over time [44] and reflects the consequences of feeding behaviour over the previous weeks [45]. What is important to underline is that when the stocking density cut-off was set to 3.95 m^2/horse, we identified a higher number of animals judged as thin in the high stocking density condition. This may mean that space allowance can also influence the time dedicated to feeding. A reduction in the feeding space would be a problem if it precludes easy access to feed, which may also increase competition for resources and thus influence the daily growth rate [32].

Of the other welfare indicators describing appropriate behaviour, "resting in a standing position" also seemed to be influenced by the stocking density. The results suggest that the number of horses resting in a standing position was significantly higher in the groups characterized by a higher stocking density (HSD^{50th} and HSD^{75th}), which could be a consequence of the lack of space and physical restriction [12]. Interestingly, the other behaviours included in the checklist (mutual grooming, resting in lying position, playing, sexual behaviour, aggressive behaviour, and stereotypic behaviour) were only detected at a very low frequency or absent altogether. However, the sampling method used may have influenced the results, as these behaviours may occur at much lower frequencies, meaning that the spot sampling method was not sensitive enough to detect their expression. The expression of certain behaviours could even be masked by the sampling method, as may be the case for stereotypic behaviour [46]. Other authors have speculated that the absence of certain behaviours might be a sign of a state of apathy [43]. Horses may be particularly sensitive to unfavorable environmental conditions, which could induce them to show apathy and become less reactive to environmental stimuli [42,47]. This condition could lead to the development of "depressive syndromes", as reported by Fureix and colleagues [48]. Studies on the behavioural repertoire of horses reared for meat production are needed to investigate this possibility. In addition to revealing conditions of negative welfare status, behavioural indicators also provide a means of recognising positive affective experiences, especially when animals exercise agency [49], which, as defined by Mellor [24], is the expression of behaviours performed by animals in a voluntary way since they are involved in rewarding experiences.

It is also important to consider the feeding management strategies used in this kind of breeding farm. Unfortunately, it was not possible to quantify the exact amount of hay supplied to the animals. Consequently, it was not possible to calculate the exact forage intake/animal/day. Nonetheless, we estimated that animals received approximately 6 kg of hay per day. Since hay was only supplied twice a day, we can presume that horses spent long periods of time fasting during the day and night. In fact, we performed the welfare assessment checklist in the morning 2 h after food provision, and we can confirm that feed bunks were not sufficiently full to guarantee an adequate provision of hay until the evening meal. Moreover, horses were fed 8 kg/animal/day of a cereal-based commercial pelleted feed that was high in starch (55% as fed). It is well known that feeding horses with high amounts of starch can affect their welfare, leading to gastrointestinal and behavioural disorders [6]. Indeed, a number of equine studies state that starch consumption should be limited to not more than 2 g starch/kg bodyweight (BW) per meal [6,38,50]—equivalent to no more than 1 kg of starch/meal for a 500 kg horse or 1820 g/meal of the commercial cereal-based pelleted feed used in the present study. At this farm, the horses received 4 kg/animal/meal of the cereal-based commercial pelleted feed, corresponding to 2.2 kg of starch/animal/meal. Although it was not possible to measure the BW of the horses involved in the present study, according to the breeder, the animals belonging to the Italian heavy draft breeds and the French heavy draft breeds weighed approximately 500 and 550 kg, respectively. Therefore, we can speculate that the amount of starch fed to the animals was approximately twice the recommended safe level.

The main limitation of the present study is related to the fact that all the assessments were made in a single farm, even though it is one of the biggest meat horse breeding farms in Italy. Moreover, it was not possible to have a control group in which the minimum requirements considered by AWIN were satisfied. Even with these limitations, the present study represents the first scientific attempt to assess the welfare of horses reared for meat production at a farm level. The data obtained show the need to understand more about the welfare of those animals, stimulating further investigations to elucidate the minimum space allowance per horse in a group pen required to generate improvements in horse welfare. Measures are also needed to improve the feeding management regimes used, which should consider the nutritional requirements and welfare of the horses and not just production goals.

5. Conclusions

Stocking densities and feeding management regimes affect the welfare of horses reared in group pens for meat production and thus constitute key concerns. The results of the present study suggest that horse welfare is negatively affected by high stocking densities and the use of an intensive feeding management strategy. According to our results, when the horses had more than 4.75 m^2/horse, many parameters were affected (i.e., improvement of coat cleanliness, improvement of bedding quantity, improvement of the mane and the tail condition, less resting in a standing position and less feeding related to the greater space available at the feed bunk). A further increment of space and/or changes in management regimes may be necessary to improve all the welfare indicators. The horses of this study were fed rations rich in starch, which was probably responsible for the high incidence of diarrhea and, consequently, the poor state of bedding cleanliness. This present study highlights the need for developing specific guidelines and rules for farming equines in order to safeguard their welfare. Moreover, since there is a lack of science-based minimum requirements for space allowance and feeding space for group-housed horses, this work hopes to stimulate and encourage further scientific inquiries into the management practices applied in horse farms for meat production.

Author Contributions: Conceptualisation: F.R., M.T., and E.V. (Emanuela Valle); data curation: F.R., C.M.M., A.V., E.V. (Ermenegildo Valvassori), and E.V. (Emanuela Valle); methodology: F.R., M.T., C.B., C.M.M., A.V., E.V. (Ermenegildo Valvassori), and E.V. (Emanuela Valle); writing of the original draft manuscript, F.R., D.B., I.V. and E.V. (Emanuela Valle); draft manuscript review and editing; F.R., M.T., D.B., I.V., and E.V. (Emanuela Valle). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QL (accessed on 19 November 2019).
2. Belaunzaran, X.; Bessa, R.J.B.; Lavín, P.; Mantecón, A.R.; Kramer, J.K.G.; Aldai, N. Horse-meat for human consumption—Current research and future opportunities. *Meat Sci.* **2015**, *108*, 74–81. [CrossRef] [PubMed]
3. Tateo, A.; De Palo, P.; Ceci, E.; Centoducati, P. Physicochemical properties of meat of Italian Heavy Draft horses slaughtered at the age of eleven months. *J. Anim. Sci.* **2008**, *86*, 1205–1214. [CrossRef] [PubMed]
4. Blanco-Penedo, I.; Ló Pez-Alonso, M.; Shore, R.F.; Miranda, M.; Castillo, C.; Herná Ndez, J.; Benedito, J.L. Evaluation of organic, conventional and intensive beef farm systems: Health, management and animal production. *Animal* **2012**, *6*, 1503–1511. [CrossRef]
5. Hartmann, E.; Søndergaard, E.; Keeling, L.J. Keeping horses in groups: A review. *Appl. Anim. Behav. Sci.* **2011**, *136*, 77–87. [CrossRef]
6. Durham, A.E. The Role of Nutrition in Colic. *Vet. Clin. N. Am. Equine Pract.* **2009**, *25*, 67–78. [CrossRef] [PubMed]
7. Spinu, M.; Benveneste, S.; Degen, A.A. Effect of density and season on stress and behaviour in broiler breeder hens Effect of density and season on stress and behaviour in broiler breeder hens. *Br. Poult. Sci.* **2003**, *44*, 170–174. [CrossRef] [PubMed]

8. Fu, L.; Li, H.; Liang, T.; Zhou, B.; Chu, Q.; Schinckel, A.P.; Yang, X.; Zhao, R.; Li, P.; Huang, R. Stocking density affects welfare indicators of growing pigs of different group sizes after regrouping. *Appl. Anim. Behav. Sci.* **2016**, *174*, 42–50. [CrossRef]
9. Telezhenko, E.; von Keyserlingk, M.A.G.; Talebi, A.; Weary, D.M. Effect of pen size, group size, and stocking density on activity in freestall-housed dairy cows. *J. Dairy Sci.* **2012**, *95*, 3064–3069. [CrossRef]
10. Benhajali, H.; Richard-Yris, M.-A.; Leroux, M.; Ezzaouia, M.; Charfi, F.; Hausberger, M. A note on the time budget and social behaviour of densely housed horses A case study in Arab breeding mares. *Appl. Anim. Behav. Sci.* **2008**, *112*, 196–200. [CrossRef]
11. Burla, J.B.; Rufener, C.; Bachmann, I.; Gygax, L.; Patt, A.; Hillmann, E. Space allowance of the littered area affects lying behavior in group-housed horses. *Front. Vet. Sci.* **2017**, *4*, 1–12. [CrossRef]
12. Yarnell, K.; Hall, C.; Royle, C.; Walker, S.L. Domesticated horses differ in their behavioural and physiological responses to isolated and group housing. *Physiol. Behav.* **2015**, *143*, 51–57. [CrossRef] [PubMed]
13. Heleski, C.R.; Nielsen, B.D.; Zanella, A.J. Influence of housing on weanling horse behavior and subsequent welfare. *Appl. Anim. Behav. Sci.* **2002**, *78*, 291–302. [CrossRef]
14. Vanhonacker, F.; Verbeke, W.; Van Poucke, E.; Buijs, S.; Tuyttens, F.A.M. Societal concern related to stocking density, pen size and group size in farm animal production. *Livest. Sci.* **2009**, *123*, 16–22. [CrossRef]
15. Veissier, I.; Butterworth, A.; Bock, B.; Roe, E. European approaches to ensure good animal welfare. *Appl. Anim. Behav. Sci.* **2008**, *113*, 279–297. [CrossRef]
16. Welfare Quality®. *Welfare Quality® Assessment Protocol for Pig (Sows and Piglets, Growing and Finishing Pigs)*; Welfare Quality®Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequality.net/media/1018/pig_protocol.pdf (accessed on 18 June 2020).
17. Welfare Quality®. *Welfare Quality® Assessment Protocol for Poultry (Broilers and Laying Hens)*; Welfare Quality® Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequalitynetwork.net/media/1019/poultry_protocol.pdf (accessed on 18 June 2020).
18. Welfare Quality®. *Welfare Quality® Assessment Protocol for Cattle*; Welfare Quality® Consortium: Lelystad, The Netherlands, 2009. Available online: http://www.welfarequalitynetwork.net/media/1088/cattle_protocol_without_veal_calves.pdf (accessed on 18 June 2020).
19. Broom, D.M. Animal Welfare in the European Union-Directorate General for Internal Policies. Policy Department C: Citizens' Rights and Constitutional Affairs Petitions. European Parliament Policy Department, Citizen's Rights and Constitutional Affairs, Study for the PETI Committee: Brussels, Belgium. Available online: https://op.europa.eu/en/publication-detail/-/publication/74df7b49-ffe7-11e6-8a35-01aa75ed71a1/language-en (accessed on 22 June 2020).
20. The Swiss Federal Council. Animal Welfare Ordinance, 455.1. (English Version) 1–152. 2008. Available online: https://www.tierschutz.uzh.ch/dam/jcr:ffffffff-eb0a-0d29-ffff-ffff8b68fdea/animal_welfare_ordinance_455_1.pdf (accessed on 22 June 2019).
21. AWIN European Union. AWIN Welfare Assessment Protocol for Horses. Available online: https://air.unimi.it/retrieve/handle/2434/269097/384836/AWINProtocolHorses.pdf (accessed on 7 March 2020).
22. Botreau, R.; Veissier, I.; Pern, P. Overall assessment of animal welfare: Strategy adopted in Welfare Quality®. *Anim. Welf.* **2009**, *18*, 363–370.
23. Brambell, F.W.R. *Report of the Technical Committee to Equire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems*, 1st ed.; AW& Co Ltd.: Hemel Hempstead, UK, 1965.
24. Mellor, D. Updating Animal Welfare Thinking: Moving beyond the "Five Freedoms" towards "A Life Worth Living". *Animals* **2016**, *6*, 21. [CrossRef]
25. Mason, G.; Mendl, M. Why is there no simple way of measuring animal welfare? *Anim. Welf.* **1993**, *2*, 301–319.
26. Bøtner, A.; Broom, D.; Doherr, M.G.; Domingo, M.; Hartung, J.; Keeling, L.; Koenen, F.; More, S.; Morton, D.; Oltenacu, P.; et al. EFSA Panel on Animal Health and Welfare (AHAW). *EFSA J.* **2012**, *10*, 2635.
27. Johnsen, P.F.; Johannesson, T.; Sandøe, P. Assessment of Farm Animal Welfare at Herd Level: Many Goals, Many Methods. *Acta Agric. Scand. A Anim. Sci.* **2001**, *51*, 26–33. [CrossRef]
28. Bertocchi, L.; Fusi, F.; Angelucci, A.; Bolzoni, L.; Pongolini, S.; Strano, R.M.; Ginestreti, J.; Riuzzi, G.; Moroni, P.; Lorenzi, V. Characterization of hazards, welfare promoters and animal-based measures for the welfare assessment of dairy cows: Elicitation of expert opinion. *Prev. Vet. Med.* **2018**, *150*, 8–18. [CrossRef]

29. National Farm Animal Care Council. Code of Practice for the Care and Handling of Equines. Available online: https://www.nfacc.ca/pdfs/codes/equine_code_of_practice.pdf (accessed on 13 May 2019).
30. Wageningen UR Livestock Research Welfare Monitoring System-Assessment protocol for horses. Available online: http://edepot.wur.nl/238619 (accessed on 13 May 2019).
31. Carroll, C.L.; Huntington, P.J. Body condition scoring and weight estimation of horses. *Equine Vet. J.* **1988**, *20*, 41–45. [CrossRef]
32. Fraser, D.; Duncan, I.J.H.; Edwards, S.A.; Grandin, T.; Gregory, N.G.; Guyonnet, V.; Hemsworth, P.H.; Huertas, S.M.; Huzzey, J.M.; Mellor, D.J.; et al. General Principles for the welfare of animals in production systems: The underlying science and its application. *Vet. J.* **2013**, *198*, 19–27. [CrossRef] [PubMed]
33. Viksten, S.M.; Visser, E.K.; Blokhuis, H.J. A comparative study of the application of two horse welfare assessment protocols. *Acta Agric. Scand. A Anim. Sci.* **2016**, *66*, 56–65. [CrossRef]
34. Webster, J. Critical control points in the delivery of improved animal welfare. *Anim. Welf.* **2012**, *21*, 117–123. [CrossRef]
35. Dalla Costa, E.; Murray, L.; Dai, F.; Canali, E.; Minero, M. Equine on-farm welfare assessment: A review of animal-based indicators. *Anim. Welf.* **2014**, *23*, 323–341. [CrossRef]
36. McGreevy, P. Ingestive behaviour. In *Equine Behavior: A Guide for Veterinarians and Equine Scientist*; Saunders Ltd.: Nottingham, UK, 2004; pp. 189–215.
37. Jørgensen, G.H.M.; Borsheim, L.; Mejdell, C.M.; Søndergaard, E.; Bøe, K.E. Grouping horses according to gender-Effects on aggression, spacing and injuries. *Appl. Anim. Behav. Sci.* **2009**, *120*, 94–99. [CrossRef]
38. Geor, R.J.; Acvim, D.; Harris, P.A.; Ecvcn, D. How to Minimize Gastrointestinal Disease Associated With Carbohydrate Nutrition in Horses. In Proceedings of the Annual Convention of the AAEP, Orlando, FL, USA, 5 December 2007; pp. 178–185.
39. Davidson, N.; Harris, P. Nutrition and Welfare. In *The Welfare of Horses*; Springer: Dordrecht, The Netherlands, 2003; pp. 45–76.
40. Ninomiya, S.; Kusunose, R.; Sato, S.; Terada, M.; Sugawara, K. Effects of feeding methods on eating frustration in stabled horses. *Anim. Sci. J.* **2004**, *75*, 465–469. [CrossRef]
41. Hammer, C.; Holzbauer, S. Equine Biological Risk Management. Iowa State University Center for Food Security and Public Health Web Site. Available online: www.cfsph.iastate.edu/BRM (accessed on 13 January 2020).
42. Pritchard, J.C.; Lindberg, A.C.; Main, D.C.J.; Whay, H.R. Assessment of the welfare of working horses, mules and donkeys, using health and behaviour parameters. *Prev. Vet. Med.* **2005**, *69*, 265–283. [CrossRef]
43. Lesimple, C. Indicators of Horse Welfare: State-of-the-Art. *Animals* **2020**, *10*, 294. [CrossRef]
44. Becvarova, I.; Pleasant, R.S.; Thatcher, C.D. Clinical Assessment of Nutritional Status and Feeding Programs in Horses. *Vet. Clin. N. Am.-Equine Pract.* **2009**, *25*, 1–21. [CrossRef]
45. Dugdale, A.H.A.; Grove-White, D.; Curtis, G.C.; Harris, P.A.; Argo, C.M.C.G. Body condition scoring as a predictor of body fat in horses and ponies. *Vet. J.* **2012**, *194*, 173–178. [CrossRef] [PubMed]
46. Martin, P.; Bateson, P. Recording methods. In *Measuring Behaviour: An Introductory Guide*, 2nd ed.; Cambridge University Press: Cambridge, UK, 1993; pp. 84–100.
47. Burn, C.C.; Dennison, T.L.; Whay, H.R. Relationships between behaviour and health in working horses, donkeys, and mules in developing countries. *Appl. Anim. Behav. Sci.* **2010**, *126*, 109–118. [CrossRef]
48. Fureix, C.; Jego, P.; Henry, S.; Lansade, L.; Hausberger, M. Towards an Ethological Animal Model of Depression? A Study on Horses. *PLoS ONE* **2012**, *7*, e39280. [CrossRef]
49. Mellor, D.J. Enhancing animal welfare by creating opportunities for positive affective engagement. *N. Z. Vet. J.* **2015**, *63*, 3–8. [CrossRef] [PubMed]
50. Julliand, V.; De Fombelle, A.; Varloud, M. Starch digestion in horses: The impact of feed processing. *Livest. Sci.* **2006**, *100*, 44–52. [CrossRef]

animals

MDPI

Article

Time-Budget of Horses Reared for Meat Production: Influence of Stocking Density on Behavioural Activities and Subsequent Welfare

Federica Raspa [1], Martina Tarantola [1], Domenico Bergero [1], Joana Nery [1], Alice Visconti [1], Chiara Maria Mastrazzo [1], Damiano Cavallini [2], Ermenegildo Valvassori [3] and Emanuela Valle [1,*]

1 Department of Veterinary Sciences, University of Turin, 10095 Grugliasco, Italy; federica.raspa@unito.it (F.R.); martina.tarantola@unito.it (M.T.); domenico.bergero@unito.it (D.B.); joana.nery@unito.it (J.N.); alice.visconti@edu.unito.it (A.V.); chiara.mastrazzo@edu.unito.it (C.M.M.)

2 Department of Veterinary Medical Sciences, University of Bologna, 40064 Ozzano dell'Emilia, Italy; damiano.cavallini@unibo.it

3 Public Veterinary Service, ASL TO5, 10023 Chieri, Italy; e.valvassori@tin.it

* Correspondence: emanuela.valle@unito.it

Received: 6 July 2020; Accepted: 29 July 2020; Published: 1 August 2020

Simple Summary: Horses reared for meat production are kept in group pens at high stocking densities. Due to the lack of scientific knowledge concerning the welfare of horses reared in this way, the aim of the present work was to assess whether their behaviours were affected by stocking density. The time-budget of the horses was also studied to evaluate if and how it differed compared with that of wild-living horses. We found that the expression of locomotion, playing, and self-grooming increased as the space allowance per horse within the group pens increased, indicating the potential to use these behaviours as indicators of positive welfare. Moreover, an altered time-budget was identified, implicating the condition of compromised welfare in these animals. Standing was the main expressed behavioural activity. A higher than usual amount of time was spent in a lying position, and a lower than usual amount of time was dedicated to feeding and locomotion. This study was the first to assess the behaviour of horses reared for meat production. The results show that more attention needs to be directed at the housing and management conditions under which horses reared for meat production are kept in in order to improve their welfare.

Abstract: Horses reared for meat production can be kept in intensive breeding farms where they are housed in group pens at high stocking densities. The present study aimed to evaluate whether the expressed behaviours correlated with stocking density, and to compare their time-budget with that of wild-living horses. An ethogram of 13 mutually exclusive behavioural activities was developed. Behavioural observations were performed over a 72 h period on group pens selected on the basis of stocking density and the homogeneity of breed, age, height at the withers, and time since arriving at the farm. Scan sampling (n = 96 scans/horse/day) was used on 22 horses. The mean frequency (%) ± standard deviation (±SD) for each behavioural activity was calculated to obtain the time-budget. The associations between time-budget and stocking density were evaluated using a bivariate analysis. The relationships were analysed by Pearson's correlation coefficient (r). Our results show that locomotion, playing, and self-grooming positively correlated with a reduction in stocking density, indicating the potential to use these behaviours as positive welfare indicators for young horses kept in group pens. The data also revealed an unusual time-budget, where the main behavioural activity expressed was standing (30.56% ± 6.56%), followed by feeding (30.55% ± 3.59%), lying (27.33% ± 2.05%), and locomotion (4.07% ± 1.06%).

Keywords: horse; behaviour; time-budget; welfare; stocking density

1. Introduction

Most of the scientific literature on horses reared for meat production is focused on the final product—the meat—in terms of its consumption [1] and nutritional values [2,3]. In contrast, there is a lack of scientific studies assessing equine faming conditions and how to safeguard horse welfare. According to Faostat data [4], more than 500,000 horses are slaughtered in Europe each year. Among the European community countries, the consumption of horse meat is limited to Spain, Italy, France, and Belgium [1,2]. However, it is reported that there are no standardised farming conditions for the breeding of the horses reared for meat production [2]. What is clear is that farms breeding horses for meat production rear young horses [5], and that these animals are often kept in intensive farming systems in order to increase meat production performances [6]. Overcrowding and high stocking densities are a concern with regard to intensive livestock farming [7]. Indeed, the European Commission has recognised that increasing the space allowance for animals kept in group pens is key to improving their welfare [8].

A high stocking density can negatively affect horse welfare, threatening the horse's physiological and behavioural needs [9]. High stocking densities lead to spatial restrictions that may prevent the animals from expressing behaviours that would otherwise be performed under more natural conditions [10]—e.g., the reduction in the expression of positive social interactions as allogrooming [11], and the reduction in the expression of feeding behaviour while exploring and moving [12,13]. The increase in the space available per animal that accompanies a reduction in a group pen stocking density has been reported to increase the expression of certain behaviours in a number of domestic species, including growing pigs [14], broiler chickens [15], and cattle [16], and is thought to reflect an improvement in their welfare state. To the best of our knowledge, no studies have evaluated to date whether an increase in the space allowance per horse kept in a group pen can generate an improvement in the behavioural indicators of positive welfare.

According to the three dimensional-concept proposed by Fraser et al. [10], which integrates the Five Freedoms [17], an animal welfare assessment needs to encompass the study of animal behaviour. This natural-living orientation represents a reference point for the Five Domains Model proposed by Mellor [18]. Accordingly, Domain 4—labelled "Behaviour"—aims at focusing attention on the environmental circumstances and their impact on the affective states experienced by animals [19]. In particular, inadequate living conditions can affect animal behaviours, leading to modifications in their time-budget and/or behavioural repertoire [20,21]. It is reported that, despite the process of domestication, horses have maintained the species-specific behaviours of their wild ancestors [22]. Studying the time-budget of horses kept in human-managed environments is, therefore, a useful tool that can help us understand their state of welfare [23].

On this basis, the first aim of the present study was to evaluate whether the behavioural activities performed by horses reared for meat production were affected by the stocking density in which they were housed. The second aim was to investigate the time-budget of horses kept in intensive breeding farms for meat production and to compare the observed time-budget with the data available in the scientific literature about wild-living horses.

2. Materials and Methods

The present study was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin, Italy (Prot. n. 2202).

2.1. Animals and Animal Husbandry

The present study was conducted in the biggest horse breeding farm for meat production in Northern Italy. This farm adopts intensive farming methods and sends a total of 2000 horses to slaughter each year. This farm housed around 300 young horses of 16 ± 8 months (mean ± standard deviation) for each cycle of production. The horses—of different heavy draft breeds and both sexes—were housed

and managed according to typical farm conditions for meat production, and none of the conditions were altered in any way for the purposes of this research. The horses were housed in group pens in a barn with two open sides, and they had no access to any outdoor paddock area. The pens were characterised by different sizes (from 14.9 to 46.5 m^2). On the basis of the pen size, the number of horses varied within each pen (from 2 to 15 horses) according to the choice of the breeder. Stallions and female horses were kept together. Each pen was enclosed by horizontal metal rail bars, and tap water was provided by a single automatic drinker. The floor was concrete and covered with barley straw bedding that was added daily (before the evening meal) by an automatic straw-dispersing tractor to cover the pen floor with a thickness of 15 cm of straw. More details on the housing and management conditions on this farm are provided by Raspa et al., 2020 [6].

Twice a day (at 7 am and at 6 pm) from the feeding lane the horses were supplied with long-stem first-cut meadow hay (6 kg/animal/day), plus 8 kg/animal/day of a cereal-based concentrate pelleted feed, labelled as follows (% of dry matter): crude protein 14.50%, ether extract 3.50%, crude fibre 5.70%, ash 6.60%; as fed: starch 55%.

2.2. Selection of Group Pens

The inclusion criteria for pen selection were based on the stocking densities. Moreover, group pens needed to be homogenous for breed, age, height at the withers, and time since arriving at the farm. This latter criterion ensured that all the horses were equally accustomed to the housing and management conditions of the breeding farm.

Stocking density was expressed as the m^2 per horse (m^2/horse). Once the area of each pen was recorded, it was divided by the mean height of the horses, measured to the withers, within the pen. A laser meter was used to measure the height of animals at the withers, and only pens containing same-sized animals were assessed. The space allowance at the feed bunk was calculated by dividing the length of the feed bunk (meters) by the number of horses within the pen (m/horse).

Only three group pens in the barn met these criteria. Table 1 reports the number of horses, pen area (m^2), stocking density (m^2/horse), and feeding space per horse at the feed bunk (m/horse) for each pen. A total amount of 22 horses (19 males and 3 females) with a height at the withers ranging between 140 and 150 cm were involved in the study. All the horses belonged to the Comtois breed, and their mean age (±standard deviation) was 22 ± 2 months. All the animals had spent six weeks in the barn before being involved in the present study.

Table 1. The number (*N*) of horses, pen area (m^2), stocking density (m^2/horse), and space at the feed bunk (m/horse) within each pen are reported.

Id Pen	*N* of Horses	Pen Area (m^2)	Stocking Density (m^2/horse)	Space at the Feed Bunk (m/horse)
A	8	35.00	4	0.88
B	8	36.75	5	0.61
C	6	36.00	6	0.80

2.3. Behavioural Observations

One 2D camera equipped with infrared light (Hikvision IP 3.0 Megapixel—NDV Network Video Recorder Hikvision 7600 Series) was installed on each selected pen. The cameras were oriented so that the horses were never out of sight. Observations were recorded for 72 h, corresponding to three consecutive days (24th to 26th November).

The videos were evaluated by two trained observers—experts in the equine field—using an ethogram recording sheet (Table 2). The ethogram was developed to assess 13 mutually exclusive behavioural activities, meaning that the horse could only be doing one of the named activities at any one time (as suggested by McFarland and Sibly, 1975 [24]). Before the behavioural data were collected, the observers underwent specific training to be ensure an adequate degree of concordance in how they

interpreted the data. Thus, the inter- and intra-observer reliability were evaluated as indicated in the data and statistical analysis section. The observations of behavioural activities were performed using scan sampling [25,26]. The behaviours expressed by each horse in the pens were assessed by scan sampling at 15 min intervals throughout the 72 h observation period.

Table 2. Description and illustrations of the selected mutually exclusive behaviour activities.

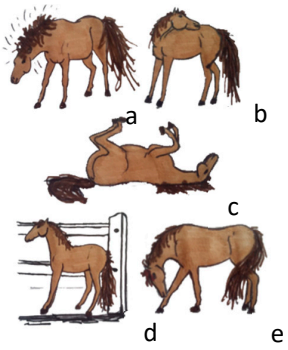
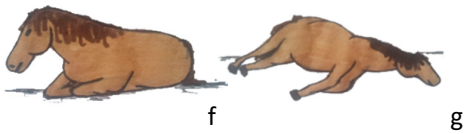
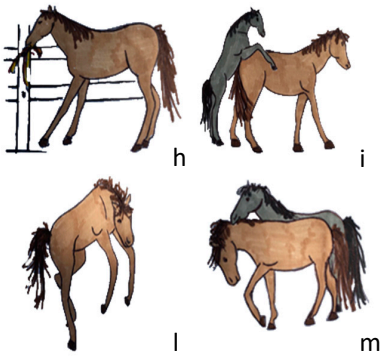
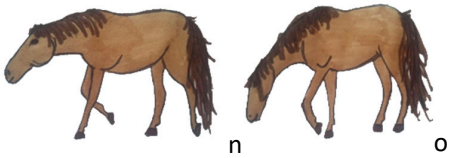
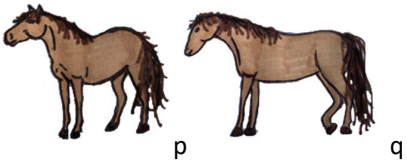
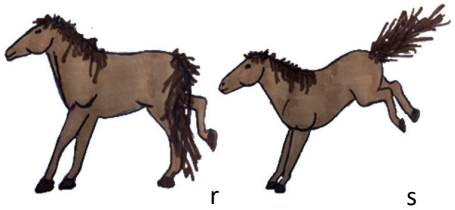
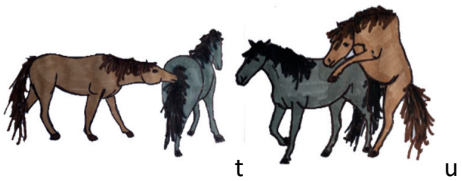
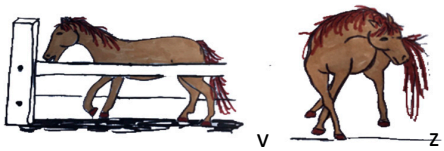
Activities	Descriptions	Illustrations
Self-grooming	The horse performs body cleaning by himself. It includes: shaking the entire body or a part of it (a); nibbling or licking the coat hair (b); rolling on the ground (c); rubbing parts of the body against objects (d) or other parts of the body (e.g., rubbing the muzzle against the limbs) (e).	
Mutual grooming	Body cleaning is performed reciprocally or by one horse towards a conspecific.	
Lying	The horse is lying on the ground in the sternal position with the limbs flexed below the body (f) or in lateral position with extended limbs (g).	
Playing	The horse plays alone or with other horses. It includes: play with structural parts of the pen (h), sexual play (i), locomotor play (l), and play fighting (m).	
Locomotion	The horse moves inside the pen by taking steps; the neck is in a horizontal position (n) or lowered to the ground to sniff (o).	

Table 2. *Cont.*

Activities	Descriptions	Illustrations
Feeding	The horse eats hay, straw or feedstuff in the trough or on the ground.	
Drinking	The horse drinks.	
Standing	The horse is in quadrupedal station. The expression is relaxed or attentive. It includes: "standing alert" (p) and "standing relaxed" (q).	 p q
Snaking	The horse stretches its neck towards a conspecific with the ears turned backwards, the lips are often closed and the body is in a dominant position.	
Kicking	The horse lifts one (r) or both hind limbs (s) off the ground and quickly stretches it/them towards a conspecific, aiming to hit him.	 r s
Biting	The horse quickly opens and closes its mouth and its teeth touch the body of a conspecific, aiming to bite him. The ears are turned backwards.	
Sexual behaviour	The stallion sniffs or bites the mare's genitals (t). The stallion mounts the mare: erection and penetration are present (u).	 t u
Stereotypic behaviour	The horse expresses a stereotyped behaviour: both oral (v) and locomotor stereotypes (z) are considered.	 v z

2.4. Data and Statistical Analysis

Statistical analyses were performed using JMP v14.3 (SAS Institute Inc., Cary, NC, USA). The inter- and intra-observer reliability of the trained observers was evaluated by means of the Cohen's Kappa Coefficient (K).

Each pen was considered as a statistical unit. In order to investigate the time-budget pattern, we used the frequency (%) ± SD for the selected behavioural activities. Frequencies were calculated for each day of observation, and data were collected for:

- 24 h periods (%/24 h);
- 12 daylight hours (8:00 am–8:00 pm) (%/daylight hours);
- 12 night hours (8:00 pm–8:00 am) (%/night hours).

2.4.1. Correlations between Time-Budget and Stocking Densities within Group Pens

Bivariate analysis was used to investigate the effect of stocking density (categorical predictors, 4, 5 and 6 m^2/horse) on the behavioural activity frequencies (%/24 h; %/daylight hours; %/night hours). Relationships were analysed using the Pearson's correlation coefficient (r, 1 or −1 depending on whether the variables are positively or negatively related [27]). The r coefficient values for correlation were interpreted according to Prior and Haerling [28]: very strong correlation (±0.91 to ±1.00); strong correlation (±0.68 to ±0.90); moderate correlation (±0.36 to ±0.67); weak correlation (±0.21 to ±0.35); and negligible correlation (0 to ±0.20). The probability of correlation (p-value) was calculated and Pearson correlations were considered significant at $p \leq 0.05$.

2.4.2. Overall Time-Budget and Time Frame

We calculated the mean frequency value for each behavioural activity for the 72 h observation period (overall time-budget) considering all 22 horses. The overall time-budget of each behavioural activity engaged in by the horses was further divided according to 6 time intervals (00:00–04:00; 04:00–08:00; 08:00–12:00; 12:00–16:00; 16:00–20:00; 20:00–24:00) as described by Boyd et al. [29]. In particular, data for the time-budget of the main expressed behavioural activities (feeding, lying, standing, and locomotion) performed by young Przewalski horses (age range: 2 to 3 years) were adapted from Boyd et al. [29] in order to compare the behavioural activities between horses reared for meat production and wild-living horses.

3. Results

The inter-observer reliability was exceptionally high: K = 0.83 (95% CI [0.72–0.94]) The intra-observer reliability was substantial K = 0.67 (95% CI [0.59–0.75]) for the first evaluator, and very high for the second evaluator K = 0.81 (95% CI [0.75–0.87]) [30].

A total amount of 96 scans per horse were performed each day, providing a total of 6336 scans sampled over the 72 h video-recordings.

3.1. Correlations between Time-Budget and Stocking Densities within Group Pens

The reduction in the stocking density and the subsequent increase in the space allowance per horse (from 4 to 6 m^2/horse) was positively correlated with locomotion ($r = 0.89$, $p = 0.001$), playing ($r = 0.73$, $p = 0.024$), and self-grooming ($r = 0.76$, $p = 0.018$) (Table 3). The data obtained revealed that the reduction in stocking density correlated with a higher frequency in the expression of these activities by horses. Locomotion showed a positive correlation with the reduction in stocking density during both the 12 daylight hours (%/12 light hours) ($r = 0.76$, $p = 0.017$) and 12 night hours (%/12 night hours) ($r = 0.67$, $p = 0.049$). Playing seemed to be positively and significantly correlated with the reduction in stocking density during the 12 daylight hours ($r = 0.79$, $p = 0.012$), but not during the 12 night hours ($r = 0.29$, $p = 0.444$); the same was true for self-grooming, which showed a positive correlation during the 12 daylight hours ($r = 0.78$, $p = 0.014$), but not during the 12 night hours ($r = 0.48$, $p = 0.193$).

Table 3. Associations between the time-budgets (%/24 h; %/12 light hours; %/12 night hours) and stocking densities among the group pens.

Behavioural Activities	Stocking Density					
	%/24 h		%/12 Light Hours		%/12 Dark Hours	
	r [a]	*p*-Value	*r* [a]	*p*-Value	*r* [a]	*p*-Value
Standing	−0.61	0.079	−0.51	0.157	−0.68	0.049 *
Feeding	−0.23	0.559	−0.14	0.724	−0.32	0.396
Lying	0.59	0.094	−0.08	0.839	0.57	0.112
Locomotion	0.89	0.001 *	0.76	0.017 *	0.67	0.049 *
Playing	0.73	0.024 *	0.79	0.012 *	0.29	0.444
Drinking	−0.29	0.450	−0.56	0.114	0.00	0.997
Snaking	0.23	0.553	0.28	0.461	0.04	0.911
Mutual grooming	0.29	0.449	0.28	0.473	0.17	0.659
Biting	0.36	0.346	0.29	0.450	0.39	0.301
Self-grooming	0.76	0.018 *	0.78	0.014 *	0.48	0.193
Kicking	0.35	0.361	0.37	0.330	0.10	0.807
Sexual behaviour	0.38	0.317	0.39	0.297	0.00	1.000
Stereotypic behaviour	0.43	0.244	0.43	0.244	0.43	0.244

[a] Pearson's correlation coefficient. * Statistical significance $p < 0.05$

Although standing was not significantly correlated with stocking density over the whole 24 h period, a negative correlation was shown during the 12 night hours ($r = -0.68$, $p = 0.049$). Based on this data, the reduction in the stocking density was associated with a reduction in the expression of standing behaviour during the 12 night hours of the 24 h period.

3.2. Overall Time-Budget and Time Frame

As represented in Figure 1, the overall time-budget of each behavioural activity engaged in by horses reared for meat production showed that the main expressed activities were: standing (30.56% ± 6.56%), feeding (30.55% ± 3.59%), and lying (27.33% ± 2.05%). Locomotion occupied only 4.07% ± 1.06% of the time. All the other activities occupied less than the 2% of the overall time-budget. In particular, stereotypic behaviours were performed the least, occupying just 0.04% ± 0.12% of the time.

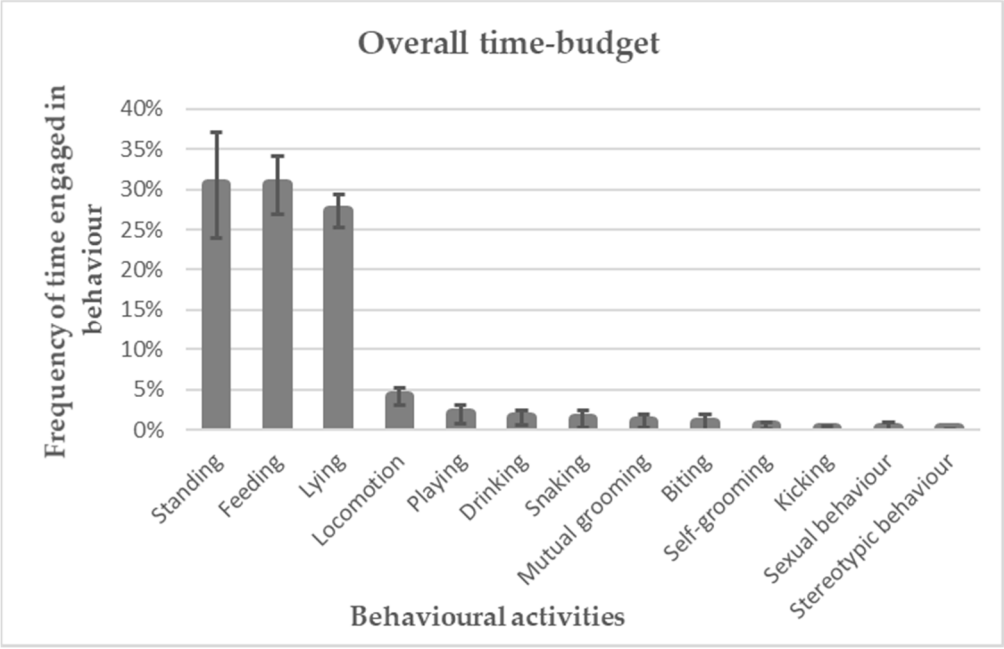
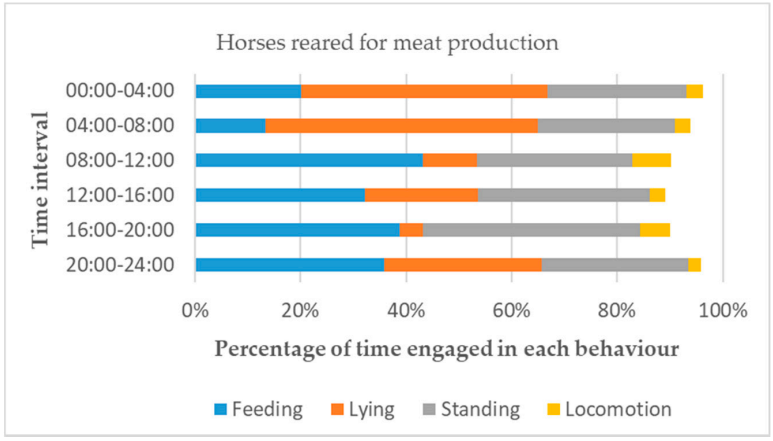


Figure 1. Frequency of time (%) spent by horses in behavioural activities.

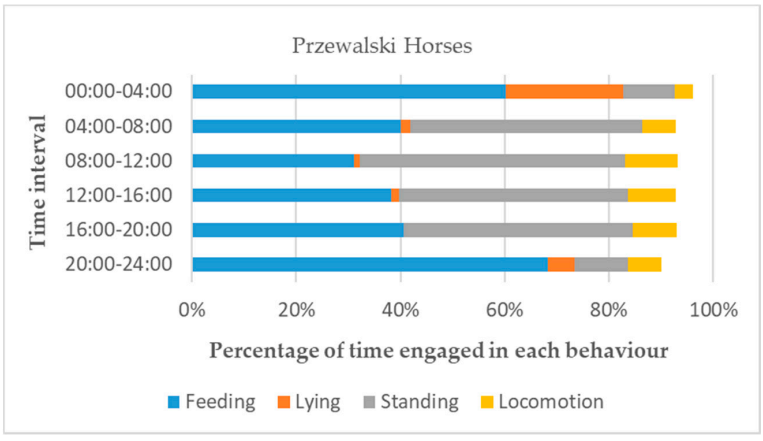
The overall time-budget of each behavioural activity shown by horses was divided into six time intervals (00:00–04:00; 04:00–08:00; 08:00–12:00; 12:00–16:00; 16:00–20:00; 20:00–24:00). As reported in Table 4, the main activity from 00:00–04:00 was lying (46.61% ± 1.19%), followed by standing (26.33% ± 4.05%), feeding (20.14% ± 2.12%), and locomotion (3.07% ± 1.63%). The time interval 04:00–08:00 showed a similar pattern, with lying being the main behaviour (51.48% ± 6.79%), followed by standing (26.01 ± 4.31%), feeding (13.43% ± 4.96 %), and locomotion (3.01% ± 0.75%). Considering the 08:00–12:00 time interval, the main activity was feeding (43.11% ± 3.65%), followed by standing (29.40% ± 6.99%), lying (10.30% ± 5.10%), and locomotion (7.38% ± 4.66%). The main activity expressed during the 12:00–16:00 time interval was standing (32.67% ± 6.93%), then feeding (31.94% ± 3.40%), lying (21.38% ± 0.93%), and locomotion (2.95% ± 0.15%). The same pattern of expression was also shown for 16:00 to 20:00, where the main expressed activity was standing (41.06% ± 1.48%), followed by feeding (38.74% ± 5.64%), locomotion (5.70% ± 4.26%), and lying (4.46% ± 2.13%). From 20:00 to 24:00, feeding was the main activity (35.94% ± 4.19%), followed by lying (29.77% ± 2.61%), standing (27.86% ± 6.64%), and locomotion (2.34% ± 1.71%).

Stereotypic behaviour was only present during the time intervals 12:00 to 16:00 and 20:00 to 24:00, although horses were only engaged in this activity for 0.12% ± 0.20% of the time.

Figure 2 shows the comparison of the 24 h time frame of the main expressed behavioural activities (standing, feeding, lying, and locomotion) performed by the horses reared for meat production and young Przewalski horses (data adapted from Boyd et al., 1988 [29]).



(**a**)



(**b**)

Figure 2. Comparison of the 24 h time frame of the main expressed behavioural activities (standing, feeding, lying, and locomotion) engaged in by the horses reared for meat production (**a**) and wild-living Przewalski horses (**b**) (data adapted from Boyd et al., 1988 [29]).

Table 4. Overall time-budget and time frames of different behavioural activities performed by horses reared for meat production. Frequencies (%) of behavioural activities are expressed as means ± SD.

Behavioural Activities (%)	Overall Time-Budget	00:00–04:00	04:00–08:00	08:00–12:00	12:00–16:00	16:00–20:00	20:00–24:00
Standing	30.56 ± 6.56	26.33 ± 4.05	26.01 ± 4.31	29.40 ± 6.99	32.67 ± 6.93	41.06 ± 1.48	27.86 ± 6.64
Feeding	30.55 ± 3.59	20.14 ± 2.12	13.43 ± 4.96	43.11 ± 3.65	31.94 ± 3.40	38.74 ± 5.64	35.94 ± 4.19
Lying	27.33 ± 2.05	46.61 ± 1.19	51.48 ± 6.79	10.30 ± 5.10	21.38 ± 0.93	4.46 ± 2.13	29.77 ± 2.61
Locomotion	4.07 ± 1.06	3.07 ± 1.63	3.01 ± 0.75	7.38 ± 4.66	2.95 ± 0.15	5.70 ± 4.26	2.34 ± 1.71
Playing	1.97 ± 1.16	0.58 ± 1.00	1.56 ± 0.90	3.36 ± 3.03	3.13 ± 1.04.	3.04 ± 2.12	0.17 ± 0.30
Drinking	1.51 ± 0.86	1.22 ± 0.91	1.19 ± 0.58	1.59 ± 1.18	2.03 ± 1.10	0.90 ± 0.62	2.17 ± 0.66
Snaking	1.27 ± 1.07	0.43 ± 0.54	1.33 ± 0.99	2.08 ± 0.90	1.24 ± 1.04	2.11 ± 1.72	0.43 ± 0.54
Mutual grooming	1.07 ± 0.85	0.69 ± 1.20	1.04 ± 0.90	1.01 ± 0.95	1.74 ± 1.59	1.56 ± 1.38	0.38 ± 0.39
Biting	0.84 ± 1.00	0.43 ± 0.54	0.78 ± 1.14	0.81 ± 0.56	1.22 ± 1.08	1.50 ± 1.12	0.29 ± 0.27
Self-grooming	0.52 ± 0.37	0.49 ± 0.43	0.17 ± 0.30	0.64 ± 0.70	0.93 ± 0.70	0.49 ± 0.22	0.41 ± 0.36
Kicking	0.19 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.18	0.55 ± 0.74	0.26 ± 0.26	0.12 ± 0.20
Sexual behaviour	0.07 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.20	0.12 ± 0.20	0.17 ± 0.15	0.00 ± 0.00
Stereotypic behaviour	0.04 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.20	0.00 ± 0.00	0.12 ± 0.20

4. Discussion

Studying the behaviours of animals reared in human-managed environments and comparing their time-budgets with those of animals living in natural environments is important for understanding animal welfare in the former [20]. Despite the process of domestication, horses have maintained the species-specific behaviours of their wild ancestors [23]. Consequently, the reduction in the horse's behavioural repertoire and/or the change in time-budget can reflect a low or inadequate welfare status [21,31].

In the present study, the daily time-budget performed by horses reared for meat production was mainly expressed by standing (30.56% ± 6.56%), feeding (30.55% ± 3.59%), and lying (27.33% ± 2.05%). Locomotion was engaged in 4.07% ± 1.06% of the time. By comparing these results with the data available in the literature about young (2–3 years old) wild-living horses, some important differences were observed. Przewalski horses spend 46.4% of the day feeding, 33.87% of the day standing, 7.4% of the day in locomotion, and 5.3% of the day lying down [29]. Duncan, in 1980 [32], reported similar data in young Camargue horses, which spend at least 56.37% of the daily time-budget engaged in feeding behaviour, 19.41% in standing behaviour, 6.97% lying down, and 5.55% of their time in locomotion, with variations according to the seasons. Taking these two studies into account, we can say that young wild-living horses have an overall time-budget in which feeding is the main expressed behavioural activity, followed by standing, lying, and locomotion. On the contrary, the daily time-budget of the horses of the present study reared for meat production involved standing as the main expressed behavioural activity, followed by feeding, lying, and locomotion. It seems that the environmental constraints imposed by the breeding farm resulted in these horses lying down more and moving less compared with Przewalski and Camargue horses.

The strong reduction in the expression of feeding behaviour is in accordance with the studies conducted by Yarnell et al., 2015 [33], and Benhajali et al., 2008 [31]. In the study by Yarnell et al., 2015 [33], horses housed in groups in a paddock area poor in grass spent 34.89% ± 14.3% of the time expressing feeding behaviour. As suggested by the same authors, this result was the consequence of the limited availability of grass. Moreover, in the study by Benhajali et al., 2008 [31], mares densely housed in paddocks were found to engage in feeding behaviour for 25.83% ± 26.80% of their time. These authors correlated this result with the lack of foraging opportunity. According to these two studies, our results could be interpreted in the same way, since animals were fed just twice a day with approximately 6 kg of hay/animal/day.

The reduction in feeding behaviour could also be linked to the lack of adequate space at the feed bunk, as shown in studies on other livestock species [34]. To this regard, the Code of Practice for the Care and Handling of Equines [35] recommends guaranteeing at least 1 m feeding space per horse under group-housing conditions and suggests having an extra feeding point available (i.e., one feeding point more than the number of horses). As shown in Table 1, none of the pens involved in the present study respected this indication.

The time spent standing by horses reared for meat production—30.56% ± 6.56%—was comparable with those reported in Przewalski horses at 33.87% [29]. In particular, our results show that a reduction in stocking density correlates with a reduction in the expression of standing behaviour during the night hours ($r = -0.68$, $p = 0.049$).

The time-budget of our study relating to lying behaviour—27.33% ± 2.05%—is in stark contrast with the data shown for wild-living horses. Yarnell et al., 2015 [33], reported their horses to spend just 0.08% ± 0.1% of the time lying down; and the mares studied by Benhajali et al., 2008 [31], never exhibited lying behaviour. From our results, it seems that the smaller pen areas may encourage horses to lie down more, also because locomotion behaviour was found to increase as space availability increased. The reduction in the expression and/or the absence of lying behaviour is widely recognised as a sign of reduced welfare in domestic species [36,37]. However, little is known about the normal lying behaviours of horses over the course of 24 h periods, or about what factors affect lying in horses [38]. Heleski et al. [39] suggested that an increase in lying behaviour in weanlings housed in stalls could

be due to boredom and the lack of possibility to perform other behaviours. Boredom and physical restriction may also be the reason for the high frequency of lying behaviour in the horses of our study. Moreover, in the present study no correlation was found between stocking density and lying behaviour frequency. Indeed, the overall increase in space allowance per horse was probably too small to allow for any differences. In fact, no guidelines or regulations are presently available for the housing and management conditions of horses reared for meat production. The only official document issued by the EU in relation to horse welfare is the Animal Welfare Indicators (AWIN) assessment protocol for horses [40]. This document is not specific for this category of horse, but it does provide indications about the space allowance for horses kept in group housing systems. In particular, horses with a height at the withers ranging from 140 to 150 cm—as those involved in our study—require at least 7 m^2/horse. None of the pens respected this indication. As a consequence, the limitation of this present study was related to the fact that it was not possible to have a control group in which the minimum space requirement considered by AWIN was satisfied. Moreover, only one camera per pen was used, even if the camera were oriented in order that horses were never out of sight. Interestingly, the reduction in the stocking density within the group pens positively correlated with an increase in behavioural activities such as locomotion, playing, and self-grooming. Thus, having more space available allowed the horses to move and play more; these results are in accordance with studies carried out on other domestic species (e.g., dairy calves [41] and growing pigs [42]).

Increased active locomotion (e.g., active walk, trot, and canter) has been identified in relation to inappropriate housing conditions [31,43]. However, in our study, the increase in space per animal was correlated with an increase in the expression of slow walking and explorative behaviour (sniffing the ground whilst walking; see Table 2).

Playing behaviour and self-grooming have been identified as potential positive welfare indicators in many species [44–46]. In particular, although growing evidence suggests that an increase in playing behaviour in adult domestic horses could be related to inappropriate living conditions [47], it seems that young horses only express playing behaviour under favourable breeding conditions [21]. Therefore, an increase in playing behaviour according to an increase in the space available could be considered as a positive welfare indicator in young horses.

Since grooming is reported to be an expression of horse welfare [48], the increase in self-grooming according to the increase in the group pen space allowance may be linked to improved welfare and could be proposed as a positive welfare indicator in this kind of breeding farm. However, the significance of self-grooming as a positive behaviour is less clear than that of mutual grooming. In fact, it seems that when horses are kept in a group, they engage more in mutual grooming [44]. However, it has also been suggested that the performance of self-grooming could be a sign of increased welfare (being a rewarding behaviour), as proposed for mutual grooming [44].

All the other behavioural activities occupied less than 7.49% of the total daily time-budget. The particularly low frequency of stereotypic behaviour is interesting to note. It is well known that an increased frequency of stereotypic behaviour may correspond with an animal's attempt to cope with an inadequate environment [49]. However, as a result of the imposed management conditions—i.e., the high stocking densities, the feeding regime used, and the impossibility to perform free movement—standing was the main expressed daily behavioural activity. Fureix et al., 2012 [50], showed that horses living under unfavourable welfare conditions can show apathy and unresponsiveness to environmental stimuli. Although in the present work it was not possible to study body position, in order to identify the apathetic state, the poor expression of stereotypic behaviours may be linked to a depressive state in these animals. The occurrence of stereotypic behaviours represents one of the most recognised behavioural indicators of welfare impairments. It could be supposed that the unusually low presence of stereotypic behaviours in horses reared for meat production could similarly reflect a condition of poor welfare. Further investigations are needed to elucidate the significance of this unexpectedly low incidence of stereotypic behaviours. Moreover, future research should investigate the importance of safeguarding the welfare of horses reared for meat production. This can also lead to

differences in meat quality traits, as reported in other livestock species [51,52], but above all it would improve the quality of life of these animals.

5. Conclusions

Considering the different factors that could affect the time-budget of horses, the reduction in stocking density had a positive impact on the expression of some behaviours, such as locomotion, playing, and self-grooming, which could be proposed as indicators of positive welfare in young horses kept in group pens. Differences in the time-budget of horses reared for meat production were found by comparing the data with those from studies on young wild-living horses (in which the main behavioural activity performed is standing). The horses reared for meat production expressed an unusual time-budget, since, compared with wild-living horses, significantly more time was spent lying down and less time was dedicated to feeding and locomotion activities. This present study stimulates further scientific studies to improve the welfare of horses reared for meat production and to obtain insight into relationships between animal welfare and meat quality, since this latter aspect represents a powerful tool to generate changes in horse meat industry practices.

Author Contributions: Conceptualization: F.R., M.T. and E.V.; data curation: F.R., C.M.M., A.V., D.C., E.V. (Ermenegildo Valvassori) and E.V. (Emanuela Valle); methodology: F.R., M.T., C.M.M., A.V., D.C., E.V. (Ermenegildo Valvassori) and E.V. (Emanuela Valle); writing of the original draft manuscript, F.R., D.B., J.N., and E.V. (Emanuela Valle); draft manuscript review and editing; F.R., M.T., D.B., J.N., and E.V. (Emanuela Valle). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Belaunzaran, X.; Bessa, R.J.B.; Lavín, P.; Mantecón, A.R.; Kramer, J.K.G.; Aldai, N. Horse-meat for human consumption—Current research and future opportunities. *Meat Sci.* **2015**, *108*, 74–81. [CrossRef]
2. Lorenzo, J.M.; Sarriés, M.V.; Tateo, A.; Polidori, P.; Franco, D.; Lanza, M. Carcass characteristics, meat quality and nutritional value of horsemeat: A review. *Meat Sci.* **2014**, *96*, 1478–1488. [CrossRef]
3. De Palo, P.; Maggiolino, A.; Centoducati, P.; Tateo, A. Slaughtering age effect on carcass traits and meat quality of italian heavy draught horse foals. *Asian-Australas. J. Anim. Sci.* **2013**, *26*, 1637–1643. [CrossRef]
4. FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QL (accessed on 19 November 2019).
5. Tateo, A.; De Palo, P.; Ceci, E.; Centoducati, P. Physicochemical properties of meat of Italian Heavy Draft horses slaughtered at the age of eleven months. *J. Anim. Sci.* **2008**, *86*, 1205–1214. [CrossRef]
6. Raspa, F.; Tarantola, M.; Bergero, D.; Bellino, C.; Mastrazzo, C.M.; Visconti, A.; Valvassori, E.; Vervuert, I.; Valle, E. Stocking Density Affects Welfare Indicators in Horses Reared for Meat Production. *Animals* **2020**, *10*, 1103. [CrossRef]
7. Blanco-Penedo, I.; Ló Pez-Alonso, M.; Shore, R.F.; Miranda, M.; Castillo, C.; Herná Ndez, J.; Benedito, J.L. Evaluation of organic, conventional and intensive beef farm systems: Health, management and animal production. *Animals* **2012**, *6*, 1503–1511. [CrossRef] [PubMed]
8. Veissier, I.; Butterworth, A.; Bock, B.; Roe, E. European approaches to ensure good animal welfare. *Appl. Anim. Behav. Sci.* **2008**, *113*, 279–297. [CrossRef]
9. Hartmann, E.; Søndergaard, E.; Keeling, L.J. Keeping horses in groups: A review. *Appl. Anim. Behav. Sci.* **2011**, *136*, 77–87. [CrossRef]
10. Fraser, D.; Weary, D.M.; Pajor, E.A.; Milligan, B.N. Milligan, & Animal Studies Repository A Scientific Conception of Animal Welfare that Reflects Ethical Concerns Recommended Citation. *Anim. Welf.* **1997**, *6*, 187–205.
11. Hemsworth, P.H.; Mellor, D.J.; Cronin, G.M.; Tilbrook, A.J. Scientific assessment of animal welfare. *N. Z. Vet. J.* **2015**, *63*, 24–30. [CrossRef] [PubMed]

12. McGreevy, P.; Berger, J.; de Brauwere, N.; Doherty, O.; Harrison, A.; Fiedler, J.; Jones, C.; McDonnell, S.; McLean, A.; Nakonechny, L.; et al. Using the Five Domains Model to Assess the Adverse Impacts of Husbandry, Veterinary, and Equitation Interventions on Horse Welfare. *Animals* **2018**, *8*, 41. [CrossRef] [PubMed]
13. McGreevy, P. Ingestive behaviour. In *Equine Behavior: A Guide for Veterinarians and Equine Scientist*; Saunders Ltd.: Nottingham, UK, 2004; pp. 189–215.
14. Fu, L.; Li, H.; Liang, T.; Zhou, B.; Chu, Q.; Schinckel, A.P.; Yang, X.; Zhao, R.; Li, P.; Huang, R. Stocking density affects welfare indicators of growing pigs of different group sizes after regrouping. *Appl. Anim. Behav. Sci.* **2016**, *174*, 42–50. [CrossRef]
15. Simitzis, P.E.; Kalogeraki, E.; Goliomytis, M.; Charismiadou, M.A.; Triantaphyllopoulos, K.; Ayoutanti, A.; Niforou, K.; Hager-Theodorides, A.L.; Deligeorgis, S.G. Impact of stocking density on broiler growth performance, meat characteristics, behavioural components and indicators of physiological and oxidative stress. *Br. Poult. Sci.* **2012**, *53*, 721–730. [CrossRef] [PubMed]
16. Fregonesi, J.A.; Leaver, J.D. I nfluence of space allowance and milk yield level on behaviour, performance and health of dairy cows housed in strawyard and cubicle systems. *Livest. Prod. Sci.* **2002**, *78*, 245–257. [CrossRef]
17. Brambell, F.W.R. *Report of the Technical Committee to Equire into the Welfare of Animals Kept under Intensive Livestock Husbandry Systems*, 1st ed.; AW& Co Ltd.: Hemel Hempstead, UK, 1965.
18. Mellor, D. Updating Animal Welfare Thinking: Moving beyond the "Five Freedoms" towards "A Life Worth Living". *Animals* **2016**, *6*, 21. [CrossRef]
19. Mellor, D.J. Operational details of the five domains model and its key applications to the assessment and management of animal welfare. *Animals* **2017**, *7*, 60. [CrossRef]
20. Dawkins, M.S. Behaviour as a tool in the assessment of animal welfare. *Zoology* **2003**, *106*, 383–387. [CrossRef]
21. Lesimple, C. Indicators of Horse Welfare: State-of-the-Art. *Animals* **2020**, *10*, 294. [CrossRef]
22. Goodwin, D. The importance of ethology in understanding the behaviour of the horse. *Equine Vet. J. Suppl.* **1999**, *28*, 15–19. [CrossRef]
23. Waran, N.K. Can studies of feral horse behaviour be used for assessing domestic horse welfare? *Equine Vet. J.* **1997**, *29*, 249–251. [CrossRef]
24. McFarland, D.J.; Sibly, R.M. The Behavioural Final Common Path. Philosophical Transactions of the Royal Society of London. *R. Soc. Stable* **1975**, *270*, 265. Available online: https://www.jstor.org/stable/2417384 (accessed on 10 June 2020).
25. Martin, P.; Bateson, P. Recording methods. In *Measuring Behaviour: An Introductory Guide*, 2nd ed.; Cambridge University Press: Cambridge, UK, 1993; pp. 84–100.
26. Altmann, J. Observational Study of Behavior: Sampling Methods. *Behaviour* **1974**, *49*, 227–266. [CrossRef] [PubMed]
27. Puth, M.T.; Neuhäuser, M.; Ruxton, G.D. Effective use of Pearson's product-moment correlation coefficient. *Anim. Behav.* **2014**, *93*, 183–189. [CrossRef]
28. Prion, S.; Haerling, K.A. Making sense of methods and measurement: Pearson product-moment correlation coefficient. *Clin. Simul. Nurs.* **2014**, *10*, 587–588. [CrossRef]
29. Boyd, L.E.; Carbonaro, D.A.; Houpt, K.A. The 24-Hour Time Budget of Przewalski Horses. *Appl. Anim. Behav. Sci.* **1988**, *21*, 5–17. [CrossRef]
30. Landis, J.R.; Koch, G.G. The Measurement of Observer Agreement for Categorical Data. *Biometrics* **1977**, *33*, 159. [CrossRef]
31. Benhajali, H.; Richard-Yris, M.-A.; Leroux, M.; Ezzaouia, M.; Charfi, F.; Hausberger, M. A note on the time budget and social behaviour of densely housed horses A case study in Arab breeding mares. *Appl. Anim. Behav. Sci.* **2008**, *112*, 196–200. [CrossRef]
32. Duncan, P. Time-budgets of Camargue horses: II. Time-budgets of adult horses and weaned sub-adults. *Bahaviour* **1980**, *72*, 26–49. [CrossRef]
33. Yarnell, K.; Hall, C.; Royle, C.; Walker, S.L. Domesticated horses differ in their behavioural and physiological responses to isolated and group housing. *Physiol. Behav.* **2015**, *143*, 51–57. [CrossRef]
34. Huzzey, J.M.; DeVries, T.J.; Valois, P.; von Keyserlingk, M.A.G. Stocking Density and Feed Barrier Design Affect the Feeding and Social Behavior of Dairy Cattle. *J. Dairy Sci.* **2006**, *89*, 126–133. [CrossRef]

35. National Farm Animal Care Council. Code of Practice for the Care and Handling of Equines. Available online: https://www.nfacc.ca/pdfs/codes/equine_code_of_practice.pdf (accessed on 13 May 2019).
36. Weeks, C.A. A review of welfare in cattle, sheep and pig lairages, with emphasis on stocking rates, ventilation and noise. *Anim. Welf.* **2008**, *17*, 275–284.
37. Vasseur, E.; Rushen, J.; Haley, D.B.; de Passillé, A.M. Sampling cows to assess lying time for on-farm animal welfare assessment. *J. Dairy Sci.* **2012**, *95*, 4968–4977. [CrossRef] [PubMed]
38. Chaplin, S.J.; Gretgrix, L. Effect of housing conditions on activity and lying behaviour of horses. *Animals* **2010**, *4*, 792–795. [CrossRef] [PubMed]
39. Heleski, C.R.; Nielsen, B.D.; Zanella, A.J. Influence of housing on weanling horse behavior and subsequent welfare. *Appl. Anim. Behav. Sci.* **2002**, *78*, 291–302. [CrossRef]
40. AWIN European Union. AWIN Welfare Assessment Protocol for Horses. Available online: https://air.unimi.it/retrieve/handle/2434/269097/384836/AWINProtocolHorses.pdf (accessed on 7 March 2020).
41. Jensen, M.B.; Vestergaard, K.S.; Krohn, C.C. Play behaviour in dairy calves kept in pens: The effect of social contact and space allowance Margit. *Appl. Anim. Behav. Sci.* **1998**, *56*, 97–108. [CrossRef]
42. Cornale, P.; Macchi, E.; Miretti, S.; Renna, M.; Lussiana, C.; Perona, G.; Mimosi, A. Non-canine Research Effects of stocking density and environmental enrichment on behavior and fecal corticosteroid levels of pigs under commercial farm conditions. *J. Vet. Behav.* **2015**, *10*, 569–576. [CrossRef]
43. Lesimple, C.; Fureix, C.; Lescolan, N.; Richard-Yris, M.-A.; Hausberger, M. Housing conditions and breed are associated with emotionality and cognitive abilities in riding school horses. *Appl. Anim. Behav. Sci.* **2011**, *129*, 92–99. [CrossRef]
44. Boissy, A.; Manteuffel, G.; Jensen, M.B.; Moe, R.O.; Spruijt, B.; Keeling, L.J.; Winckler, C.; Forkman, B.; Dimitrov, I.; Langbein, J.; et al. Assessment of positive emotions in animals to improve their welfare. *Physiol. Behav.* **2007**, *92*, 375–397. [CrossRef]
45. Napolitano, F.; Knierim, U.; Grass, F.; De Rosa, G. Positive indicators of cattle welfare and their applicability to on-farm protocols. *Ital. J. Anim. Sci.* **2009**, *8*, 355–365. [CrossRef]
46. Held, S.D.E.; Spinka, M. Animal play and animal welfare. *Anim. Behav.* **2011**, *81*, 891–899. [CrossRef]
47. Blois-Heulin, C.; Rochais, C.; Camus, S.; Fureix, C.; Lemasson, A.; Lunel, C.; Bezard, E.; Hausberger, M. Animal welfare: Could adult play be a false friend? *Anim. Behav. Cogn.* **2015**, *2*, 156–185. [CrossRef]
48. Goodwin, D. Horse behaviour: Evolution, Domestication and Feralisation. In *The Welfare of Horses*; University of Edinburgh, Edinburgh: Scotland, UK, 2003; pp. 1–18.
49. Cooper, J.J.; Albentosa, M.J. Behavioural adaptation in the domestic horse: Potential role of apparently abnormal responses including stereotypic behaviour. *Livest. Prod. Sci.* **2005**, *92*, 177–182. [CrossRef]
50. Fureix, C.; Jego, P.; Henry, S.; Lansade, L.; Hausberger, M. Towards an Ethological Animal Model of Depression? A Study on Horses. *PLoS ONE* **2012**, *7*, e39280. [CrossRef] [PubMed]
51. Xiccato, G.; Trocino, A.; Queaque, P.I.; Sartori, A.; Carazzolo, A. Rearing veal calves with respect to animal welfare: Effects of group housing and solid feed supplementation on growth performance and meat quality. *Livest. Prod. Sci.* **2002**, *75*, 269–280. [CrossRef]
52. Paranhos Da Costa, M.J.R.; Huertas, S.M.; Gallo, C.; Dalla Costa, O.A. Strategies to promote farm animal welfare in Latin America and their effects on carcass and meat quality traits. *Meat Sci.* **2012**, *92*, 221–226. [CrossRef] [PubMed]

Received: 27 April 2021 | Accepted: 8 September 2021

DOI: 10.1111/jpn.13643

ORIGINAL ARTICLE

Journal of Animal Physiology and Animal Nutrition | WILEY

A Fibre- vs. cereal grain-based diet: Which is better for horse welfare? Effects on intestinal permeability, muscle characteristics and oxidative status in horses reared for meat production

Federica Raspa[1] | Francesca Rita Dinardo[2] | Ingrid Vervuert[3] | Domenico Bergero[1] | Maria Teresa Bottero[1] | Daniele Pattono[1] | Alessandra Dalmasso[1] | Marica Vinassa[1] | Ermenegildo Valvassori[4] | Elena Bruno[5] | Pasquale De Palo[2] | Emanuela Valle[1]

[1]Department of Veterinary Sciences, University of Turin, Grugliasco, Italy

[2]Department of Veterinary Medicine, University of Bari, Valenzano, Italy

[3]Institute of Animal Nutrition, Nutrition Diseases and Dietetics, Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany

[4]Public Veterinary Service, ASL TO5, Chieri, Italy

[5]Public Veterinary Service, ASL CN2, Alba, Italy

Correspondence
Emanuela Valle, Department of Veterinary Sciences, University of Turin, 10095 Grugliasco, Italy.
Email: emanuela.valle@unito.it

Abstract

Horses reared for meat production are fed high amounts of cereal grains in comparison with horses raised for other purposes. Such feeding practice may lead to risk of poor welfare consequences. The aim of this study was to investigate the effects of two feeding practices on selected metabolic parameters and production aspects. Nineteen Bardigiano horses, 14.3 ± 0.7 months of age, were randomly assigned to two groups—one fed with high amounts of cereal grains (HCG; $n = 9$; 43% hay plus 57% cereal grain-based pelleted feed) vs. one fed with high amounts of fibre (HFG; $n = 10$; 70% hay plus 30% pelleted fibrous feed)—for 129 days. At slaught on abattoir, biological and tissue samples were collected to evaluate the microbiological contamination of mesenteric lymph nodes and liver; selected meat quality traits (chemical composition and fatty acid profile of the *Longissimus thoracis et lumborum* muscle); and the oxidative status of the horse. A linear mixed model was used: dietary treatment and sex were fixed effects and their interaction analysed on production and metabolic parameters as dependent variables. Results showed an increased intestinal permeability in the horses fed HCG compared to HFG, according to the significant increased total mesophilic aerobic bacteria counts in mesenteric lymph nodes ($p = 0.04$) and liver samples ($p = 0.05$). Horses in HCG showed increased muscle pH ($p = 0.02$), lighter muscle colour (L) ($p = 0.01$), increased intramuscular fat concentrations ($p = 0.03$), increased muscle glutathione peroxidase and superoxide dismutase activities ($p = 0.01$ and $p = 0.03$, respectively). Moreover, horses in HCG had lower muscle water holding capacity at interaction with sex ($p = 0.03$, lower in female), lower muscle protein content ($p = 0.01$), lower concentration of muscle PUFAs ($p = 0.05$) and lower plasma catalase activities ($p = 0.05$). Our results showed that feeding a high cereal grains diet

can have global effects on horse physiology, and thus represents a threat for their welfare.

KEYWORDS
horse, intestinal permeability, *Longissimus thoracis et lumborum* muscle, nutrition, oxidative status, welfare

1 | INTRODUCTION

Animal welfare is a complex and multidimensional concept. The feeding practice adopted for horses can affect the welfare of these animals through their direct effects on the animals' health as well as by influencing horse behaviour (Lesimple, 2020). Accordingly, horses are grazing animals, adapted to eating forages. Thus, a fibre-based diet should represent the basis of horse nutrition, respecting the innate herbivorous nature of these animals (Davidson & Harris, 2007). Forages are high in structural carbohydrates and provide at least 50%–70% of a horse's energy requirements through the metabolism of volatile fatty acids (VFA) produced by bacterial fermentation in the hindgut (Merritt & Julliand, 2013). However, due to the demands placed on horses for competitions and/or productive performances (i.e. sport horses and horses destined to meat production), they are often fed with high amounts of energy-dense feedstuffs rich in hydrolysable carbohydrates, such as starch and simple sugars (Julliand et al., 2006; Raspa, Tarantola, Bergero, Bellino, et al., 2020; Raspa, Tarantola, Bergero, Nery, et al., 2020; Williamson et al., 2011). A number of studies concerning equine nutrition state that starch consumption should be limited to no more than 2 g starch/kg bodyweight (BW)/meal (Durham, 2009; Geor & Harris, 2007; Julliand et al., 2006). Feeding horses with diets characterised by a high starch content can negatively affect their welfare, increasing the risk for gastrointestinal disorders such as colic and gastric ulcers (Durham, 2009; Hudson et al., 2001). In particular, when it reaches the hindgut, the high starch content of a cereal grain-based diet causes microbiome alterations, leading to an increase in lactic acid production and a drop in pH with subsequent acidosis (Geor & Harris, 2007; Merritt & Julliand, 2013). Acidosis is reported to cause severe damage to the intestinal epithelium, leading to hyperpermeability—also known as 'leaky gut' (Stewart et al., 2017). Alterations in intestinal permeability can also lead to the translocation of enteric bacteria and/or their products from the gut lumen into the mesenteric lymph nodes and the portal circulation (Davis et al., 2003; Stewart et al., 2017), with the potential for systemic consequences. A high cereal grain intake has also been associated with several muscular disorders, such as exertional rhabdomyolysis and polysaccharide storage myopathy (PSSM), shown to result from excessive glycogen storage within the muscle (MacLeay et al., 1999; Valberg et al., 1999). Moreover, the ingestion of excessive amounts of rapidly fermentable carbohydrates has been associated with the condition of oxidative stress in horses, and biomarkers of oxidative stress have been proposed as indicators of animal welfare (Celi & Gabai, 2015).

Among the various animal species reared for meat production, also horses reared for this purpose are fed high amounts of cereal grains as a fundamental energy source (Cappai et al., 2013; Lorenzo et al., 2014; Raspa, Tarantola, Bergero, Bellino, et al., 2020; Raspa, Tarantola, Bergero, Nery, et al., 2020). Most scientific studies on the subject report that farms breeding horses for meat mainly rear young horses (Tateo et al., 2008) and that feeding regimes, which include hay plus high amount of cereals (7–8 kg/horse/day; Franco et al., 2013; Lorenzo et al., 2014; Raspa, Tarantola, Bergero, Bellino, et al., 2020; Raspa, Tarantola, Bergero, Nery, et al., 2020; Sarriés & Beriain, 2005) are primarily geared towards fattening the horses.

On such a basis, in view of the fact that nutrition can impact both on animal health and welfare, the aim of the present study was to compare the effects of two different feeding regimes—high cereal grains vs. high fibre—on production and metabolic parameters.

For these reasons, microbiological contamination of mesenteric lymph nodes and liver as potential indicators of altered intestinal permeability have been investigated by two microbiological criteria (Total Mesophilic Aerobic Bacteria counts [TMABc] and Enterobacteriaceae counts) and tested for the presence of pathogenic bacteria (*Salmonella* spp. and *Escherichia coli*). Moreover, selected meat quality traits (chemical composition and the fatty acid profile of *Longissimus thoracis et lumborum* muscle) were evaluated. Finally, horses were investigated for oxidative status by means of antioxidant enzymes and oxidation end-products determined in different biological fluids and tissues.

2 | MATERIALS AND METHODS

The present study was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy, Prot. n. 2202/2019). The study was carried out on the biggest horse farm in Northern Italy, which rears horses with the specific intention of fattening them for meat production. The housing and management features of this farm have previously been described in recent papers published by Raspa, Tarantola, Bergero, Bellino, et al., (2020); Raspa, Tarantola, Bergero, Nery, et al., (2020).

2.1 | Animals and stable features

Nineteen horses of the Bardigiano breed (12 females and 7 males) aged 14.3 ± 0.7 months (mean ± standard deviation, SD) were

treated against internal parasites (1.29 g/100 kg BW; Equalan duo; Merial Animal Health) upon arrival at the farm. During the subsequent 2 weeks, horses were kept together in an outdoor dry lot and fed the same grass hay containing mainly *Lolium Italicum* which was provided ad libitum. After the adaptation period, horses were housed in group pens in a barn with two open sides and no access to any outdoor paddock area. Horses were randomly divided into two group pens (7 x 9 m), which assured a space allowance of at least 6 m^2 per animal. The group pens were located side by side, each of which was enclosed by horizontal metal rail bars, delimiting the pens at the feed bunk level. Each pen contained a single automatic drinker providing tap water. One flake of fresh barley straw bedding was distributed across over the permanent bedding once a day before the evening meal by means of an automatic straw-dispersing tractor. Animals were weighed at the beginning and at the end of the trial in order to calculate the average daily gain in bodyweight. All horses were weighed at the same time of the day when they arrived on the farm and the evening before slaughter after the evening meal.

2.2 | Diets

The animals were randomly assigned to the two groups and they received the same hay (described in Table 1) but a different concentrate feed. One group of horses was individually fed with a high starch and sugar cereal grain-based complementary feed (HCG; 43% hay plus 57% cereal grain-based pelleted feed); the other group was individually fed with a fibre-rich complementary feed (HFG; 70% hay plus 30% pelleted fibrous feed). The composition of the different complementary feed used is provided in Table 1.

For the HCG (5 females and 4 stallions), the amount of the complementary feed used was gradually increased over a time: for the first 13 days, they received 3 kg/animal/day, followed by 4.5 kg/animal/day for the subsequent 6 days, and 5 kg/animal/day for a further 36 days; during the final part of the trial, the animals were fed 8 kg/animal/day until the end of the fattening period (72 days). Those quantities were decided by the breeder according to his conventional management system adopted in his farm (Tables 1 and 2).

For the HFG (7 females and 3 stallions), horses were fed the pelleted fibrous feed which was gradually increased over a time: 1 kg/animal/day for 7 days, 2 kg/animal/day for 9 days, 2.5 kg/animal/day for 25 days, 3 kg/animal/day for 9 days, and finally 3.5 kg/animal/day until the end of the fattening period (72 days). Those quantities were decided by the researchers according to the nutritional requirements of horses as suggested by the French Institute National de la Recherche Agronomique (INRA) (Martin-Rosset, 2015; Tables 1 and 2). The complementary feed was individually supplied to the horses twice a day (07:00 and 18:00). At the same time, hay was provided and the hay consumption was estimated to be fed 6 kg/animal/day for the HCG and 8 kg/animal/day for the HFG.

Feeds were weighed before each provision to horses and left over were monitored throughout the duration of the trial.

TABLE 1 Chemical composition (% as fed) of hay and pelleted feed

	Hay	Cereal grain-based pelleted feed HCG[a]	Pelleted fibrous feed HFG[b]
DM[c]	89.81	89.91	90.59
Crude protein	6.62	14.21	19.77
Ether extract	1.03	3.69	5.06
Crude fibre	30.04	4.44	11.53
Ash	6.23	8.30	10.78
Starch	0.27	49.50	19.11
NDF[d]	55.20	17.62	27.10
ADF[e]	35.06	6.44	15.28
ADL[f]	4.01	0.73	1.98

[a]High cereal grains group ($n = 9$).
[b]High fibre group ($n = 10$).
[c]Dry matter.
[d]Neutral detergent fibre.
[e]Acid detergent fibre.
[f]Acid detergent lignin.

2.3 | Slaughter procedures and sample collection

At the end of the fattening period (day 129), all animals were slaughtered. The commercial authorised abattoir was 7 km from the horse farm and took less than 25 min travelling time to reach. All the procedures carried out during this phase were supervised by the official veterinarian and conducted according to the European Union regulations (EU Regulation 2009/853 and EU Regulation 627/2019). After slaughtering, selected biological samples were collected as listed below.

Blood samples were collected from the jugular vein by venipuncture into tubes containing EDTA and transported to the laboratory within one hour. Blood plasma was separated by centrifugation at 1500 *g* for 10 min. Aliquots were stored at −20°C for the subsequent analysis of antioxidant enzymes and oxidation end-products as described below in Section 2.4.

Liver tissue and mesenteric lymph nodes were aseptically collected from the packed viscera immediately after evisceration by a trained operator and placed into sterile bags. Samples were transported to the laboratory at 4°C for microbiological analysis and processed within one hour. A 100 g liver sample was frozen at −20°C for subsequent analysis of antioxidant enzymes and oxidation end-products as described in Section 2.4. A 100 g liver sample and 100 g of mesenteric lymph nodes were immediately processed to assess their microbiological contamination, as described below in Section 2.5.

The *Longissimus thoracis et lumborum* muscle of the right half-carcass was immediately refrigerated at 4°C and sampled at the 17/18th thoracic vertebrae level after 24 h of storing at low temperature. One sample was processed for the analyses of muscle characteristics as described below in Sub Section 2.6.1; and one

TABLE 2 Overall nutritional composition of the diets (referred to the total daily diet: hay plus pelleted feed) as fed to the high cereal grains group (HCG) and the high fibre group (HFG) during the fattening period (72 days)

Nutritional components	HCG[a]	HFG[b]
Kg hay/animal/day	6	8
Kg pelleted feed/animal/day	8	3.5
Forage intake/kg BW (%)	1.73	2.32
DM intake (kg)	12.60	10.25
Net energy (MJ)[c]	95.88	53.58
Crude protein (g)	1557.20	1159.60
Digestible Crude Protein (g MADC)	1177.66	723.25
Crude fat (g)	285.40	192.70
Fat contribution to total energy content provided (%)	8.39	10.14
Calcium (g)	377.80	108.22
Phosphorous (g)	188.60	35.79
Lysine (g)	48	76.50
Vitamin E (mg)	399.68	1105
Selenium (mg)	0.48	1.72

[a]High cereal grains group ($n = 9$).

[b]High fibre group ($n = 10$).

[c]Net energy was calculated according to Martin-rosset, 2015.

aliquot was stored at −20°C until the subsequent analysis of its chemical composition and fatty acid profile as described below in Sub Sections 2.6.2 and 2.6.3, respectively.

2.4 | Analysis of antioxidant enzymes and oxidation end-products

Plasma, liver and muscle samples were analysed for the following antioxidant enzymes: glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), according to the methods described by Tufarelli et al. (2016) and Tateo et al. (2020). The following oxidation end-products were also determined in plasma and muscle samples: thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (HY) and dinitrophenylhydrazine (DNPH) as carbonylated proteins (PC), according to the methods described by De Palo et al. (2018).

2.4.1 | Analysis of thiobarbituric acid reactive substances (TBARs), protein carbonyls and hydroperoxides in plasma

Thiobarbituric acid reactive substances (TBARs) were measured fluorometrically according to Gondim et al., (2009), by adding 100 ml plasma to a 0.37% thiobarbituric acid solution. Plasma reactive carbonyl derivative (RCD) levels were measured according to Faure & Lafond (1995). RCD levels were determined by carbonyl reagent DNPH. Plasma (200 ml) was mixed with 1 ml water and 2 ml 20% trichloroacetic acid and centrifuged at 1000× *g* for 10 min. The pellet was resuspended in 1 ml of 10 mmol/L DNPH and incubated for 60 min at 37.8°C. In the control condition, 1 ml of 1 mol/L hydrochloric acid was used instead of DNPH. Subsequently, 1 ml of 20% trichloroacetic acid was added, and the sample was centrifuged at 1000× *g* for 10 min. The pellet was washed with 1:1 ethanolethyl acetate solution and centrifuged at 1000× *g* for 10 min. The pellet was mixed with 1 ml of 6 mol/L guanidine (diluted in 20 mmol/L dihydrogenphosphate at pH 2.3). Finally, the sample was incubated for 40 min at 37.8°C. The absorbance was measured at 380 nm. Hydroperoxides were analysed according to (Södergren et al., 1998). Aliquots (90 ml) of plasma were transferred into eight microcentrifuge vials (1.5 ml). Ten microliters of 10 mmol/L TPP in methanol were added to four of the vials to reduce ROOHs, thereby generating a quadruplicate of blanks. Methanol (10 ml) was added to the remaining four vials to produce a quadruplicate of test samples. All vials were then vortexed and incubated at room temperature for 30 min prior to the addition of 900 ml of FOX2 reagent. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2400× *g* for 10 min with a swing-out rotor (Hettich Rotenta/RP centrifuge, Hettich-Zentrifuge). Absorbance of the supernatant was measured at 560 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech). ROOH concentration in the plasma samples was calculated using the mean absorbance difference between quadruplicates of test samples and blank samples.

2.4.2 | Muscle thiobarbituric acid reactive substances (TBARs), protein carbonyls and hydroperoxides analyses

Minced muscle samples (5 g) were placed in a 50 ml test tube and homogenised with 15 ml deionised distilled water (DDW). Samples were treated as described by Maggiolino et al. (2020). The concentration of TBARS was calculated by comparison against a standard curve constructed using 1,1,3,3-tetramethoxypropane, and the concentration of lipid oxidation was expressed as milligrams of malondialdehyde (MDA) per kg of meat. Two milliliters of homogenate (previously prepared for TBARS determination) was used for hydroperoxide quantification as described by De Palo, Maggiolino et al. (2014); De Palo, Tateo et al. (2014). Results were expressed in micromoles per gram. Meat samples (2 g) were homogenised in 20 ml of 0.15 mol/L KCl for 2 min and analysed for the quantification of protein carbonyls as described by De Palo et al. (2013a).

2.5 | Procedures to assess microbiological contamination of mesenteric lymph nodes and liver samples

Mesenteric lymph nodes were processed as described by Webb et al. (2017) and Mainar-Jaime et al. (2013). Accordingly, samples of

mesenteric lymph nodes were aseptically trimmed to remove excess fat and fascia. The trimmed lymph nodes were submerged into boiling water for 3–5 s and then flamed using a Bunsen burner for 3 s. Then, they were sterile cut and weighed to obtain 25 g/animal for the detection of *Salmonella* spp., and 10 g/animal for the detection of *E. coli*.

Liver samples were surfaced flamed before proceeding with deep subsampling. Liver subsamples were then obtained using a sterile scalpel by cutting deep into the organ's tissue. Samples weighing 25 g/animal and 10 g/animal were used for the detection of *Salmonella* spp. and *E. coli*, respectively. Subsequently, samples were homogenised according to the analyses described in the subsequent sections.

2.5.1 | Total mesophilic aerobic bacteria counts and Enterobacteriaceae counts

ISO procedures were used for TMABc and Enterobacteriaceae counts (ISO 4833–1:2013 and ISO 21528–2:2017, respectively). Briefly, for the detection of TMAB, tissue samples were diluted in Buffered Peptone Water (BPW; CM 509 B, Oxoid) and appropriately plated onto Plate Count Agar (PCA CM 0325 Oxoid), then incubated at 31°C for 48 h. For the detection of Enterobacteriaceae, Violet Red Bile Glucose Agar (VRBG agar CM 0485 Oxoid, Rodano, Milan) was streaked and incubated at 37°C for 48 h. The results are expressed in CFU/g.

2.5.2 | Isolation of *Salmonella* spp

The isolation of *Salmonella* spp. was carried out in accordance with ISO 6579–1:2017. After pre-enrichment in BPW for 24 h at 37°C, 1 and 0.1 ml of each pre-enrichment solution was inoculated into 10 ml of Selenite Cystine Broth base (CM 0699, Oxoid) and 10 ml of Rappaport-Vassiliadis Broth (CM 669 B, Oxoid), respectively, and then incubated at either 37°C (Selenite Cystine Broth) or 41°C (Rappaport-Vassiliadis Broth) for 24 h and plated onto selective Xylose Lysine Deoxycholate (XLD) Agar (CM 0469, Oxoid) and Hektoen Enteric Agar (HEA) (CM 0419, Oxoid). Following 24 h incubation, suspect colonies of *Salmonella* spp. were tested by inoculation into Kligler iron agar (CM0033, Oxoid).

2.5.3 | Isolation of *Escherichia coli*

The isolation of *E. coli* spp. was performed as described in ISO 16649–12:2001 using tryptone bile x-glucuronide (TBX) medium (Oxoid Ltd). Plates were incubated at 41°C per 24 h. Suspected colonies of *E. coli* spp. were then tested using API 20 Enterobacteriaceae (API 20E) strips (BioMérieux).

2.6 | Analysis of *Longissumus thoracis et lumborum* muscle samples

2.6.1 | Muscle characteristics

Forty-eight hours after slaughtering, the rheological characteristics of muscle samples were assessed. pH measurement was performed using a portable pH meter with a glass electrode shaped to facilitate meat penetration (Carlo Erba pH 710; Carlo Erba Reagenti). Before each measurement, the pH meter was automatically calibrated for muscle temperature and using pH 4 and pH 7 buffered solutions (Crison).

The colour of *Longissimus thoracis et lumborum* muscle samples was determined according to the CIE (Comission Internationale de l'Eclairage) colour system. A Minolta CR-300 colorimeter (light source D65; Minolta Camera Co. Ltd.) was used according to the method described by De Palo et al. (2015). Forty-eight hours after slaughtering, measurements were performed on fresh samples (L a b) and then on thawed samples (L* a* b*) in three different points. At each point, measurements were performed in triplicate, making a total of nine measurements per sample, according to the method described by De Palo et al. (2017). The colorimeter was calibrated according to the Hunter-lab colour space system using a white title (L* = 99.2, a* = 1.0, b* = 1.9). The a* and b* values were used to determine chroma = $(a^2 + b^2)^{1/2}$ and hue (°) = tan−1(b/a) according to De Palo et al., 2012. Water holding capacity, thawing losses and cooking losses were measured as described by De Palo, Maggiolino et al. (2014); De Palo, Tateo et al. (2014). The concentration of haem pigment was determined according to Hornsey (1956). Results are presented as μg of acid haematin/g of muscle wet weight.

2.6.2 | Chemical composition

After thawing, samples of *Longissimus thoracis et lumborum* muscle were placed in an oven at 105°C until a constant weight was reached in order to determine moisture content. The protein content was measured according to ISO 937:1978. Intramuscular fat (IMF) was measured according to ISO 1443:1973. Each muscle was homogenised in a chloroform:ethanol solution (1:2, vol/vol) prior to the extraction of total lipids from IMF, performed using the method described by De Palo et al. (2016). Ash content was calculated according to ISO 936:1998.

2.6.3 | Fatty acid profile

According to the methods described by De Palo et al. (2015, 2016), fatty acid methyl esters (FAME) were prepared by transesterification using methanol in the presence of 3% hydrochloric acid in methanol (vol/vol). FAME were determined using a Trace GC Thermo Quest Gas Chromatograph (Thermo Electron, Rodano) equipped with a flame ionisation detector. The derivatives were separated on a capillary column (Supelco SP-2380 fused-silica column, 120 m length, 0.25 mm internal

diameter and 0.20 mm film thickness). The injector and the detector temperatures were held at 260°C. Column oven program temperatures were as follows: T1 = 80°C, hold 1 min; T2 = 150°C, ramp at 15°C/min, hold 2 min; T3 = 220°C, ramp at 5°C/min, hold 2 min; T4 = 250°C, ramp at 15°C/min, hold 5 min. The flow rate of the carrier gas (He) was set at 0.8 ml/min. FAME identifications were based on the retention times of reference compounds (Sigma-Aldrich) and mass spectrometry. Fatty acid composition was expressed as the percentage of total FAME.

The amount of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n–3 and n–6 fatty acids, SFA/UFA, SFA/MUFA and SFA/PUFA were calculated to assess nutritional implications. Finally, atherogenic and thrombogenic indices were calculated according to the formulas provided by De Palo et al. (2017):

Atherogenic index (AI) = (C12:0 + 4 x C14:0 + C16:0) / [ΣMUFA + ΣPUFA (n–6) and (n–3)]

Thrombogenic index (TI) = (C14:0 + C16:0 + C:18) / [0.5ΣMUFA + 0.5ΣPUFA (n–6) +3ΣPUFA(n–3) + (n–6)/(n–3)]

2.7 | Statistical analysis

Data were statistically analysed using the software JMPpro v15 (SAS Institute). Each parameter was tested for normal distribution using the Shapiro–Wilk test and normalised, when necessary, by box-cox transformation. A linear mixed-effects model was constructed, and the model fixed effects were the dietary treatment, the sex and their interaction. Then, each horse within sex and diet was considered as experimental unit and used as random variable for all analyses. The initial BW was set as a covariate for the slaughter BW model. Least squares means were separated using *T*-Student's adjusted *p*-values when at least a tendency F-test ($p \leq 0.10$) was detected in the fixed effect interaction term.

3 | RESULTS

3.1 | Animals

Table 3 reports the mean (SEM) initial bodyweight (iBW) of the horses of each group upon their arrival at the farm, the mean (SEM) slaughter bodyweight at end of the study (sBW) and the calculated average (SEM) daily bodyweight gain (ADG) for the two groups (HCG and HFG).

No differences in sBW according to diet, sex or their interaction were evident between the two groups of horses at the end of the trial. Moreover, ADG showed no differences in the two groups of horses according to dietary treatment, sex or their interaction.

3.2 | Microbiological contamination of mesenteric lymph nodes and liver samples

As shown in Table 4, TMABc were found increased in HCG than in HFG for both mesenteric lymph nodes ($p = 0.04$) and liver samples ($p = 0.05$), indicating a different microbial contamination in those tissues according to the dietary treatment. No differences between HCG and HFG were found in mesenteric lymph nodes ($p = 0.31$) and liver samples ($p = 0.11$) for Enterobacteriaceae counts. Moreover, no samples were found to be contaminated by *Salmonella* spp. or *E. coli*.

3.3 | Muscle characteristics and chemical composition of Longissimus thoracis et lumborum muscle

Table 5 shows the mean values (SEM) of the muscle characteristics and the chemical composition of the *Longissimus thoracis et lumborum muscle* samples obtained from horses reared using the two different feeding strategies (HCG vs. HFG). The pH was lower in HCG vs. HFG according to diet ($p = 0.02$). Water holding capacity was lower in HCG vs. HFG according to the dietary treatment ($p = 0.04$). Moreover, this latter finding resulted to be affected by the sex of the animals ($p = 0.03$) since *Longissimus thoracis et lumborum muscle* from females in HCG showed lower water holding capacity than that of females in HFG. Moreover, muscle colour in HCG was characterised by increased lightness (*L*) ($p = 0.01$) compared with muscle samples from HFG. Regarding the chemical composition of the muscle, lower moisture content ($p = 0.03$), increased protein content ($p = 0.01$) and increased concentration of intramuscular fat (IMF; ($p = 0.03$) was found in muscle samples from horses in HCG compared with those from HFG according to the dietary treatment. No differences were observed in ash concentration between the two groups.

TABLE 3 Mean (SEM) initial bodyweight (iBW), mean (SEM) slaughter bodyweight (sBW) at the end of the trial (129 days) and the calculated mean (SEM) daily bodyweight gain (ADG) for the two groups (HCG and HFG)

	HCG[a]		HFG[b]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
iBW	216.6 (4.02)	218.75 (5.44)	222 (2.07)	219 (2.08)	-	-	-
sBW	346.6 (2.42)	349 (4.38)	343.43 (0.92)	346.67 (1.76)	0.14	0.22	0.61
ADG	1.01 (0.03)	1.01 (0.03)	0.94 (0.02)	0.99 (0.02)	0.15	0.20	0.57

[a]High cereal grains group ($n = 9$).

[b]High fibre group ($n = 10$).

TABLE 4 TMABc (Total mesophilic aerobic bacteria counts) and Enterobacteriaceae counts (HCG vs. HFG): median values (plus 25th–75th percentiles) expressed as CFU/g

		HCG[a]		HFG[b]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
Mesenteric lymph nodes	TMABc	$36*10^2$ ($7*10^2$–$83.75*10^2$)	$4*10^2$ ($1.75*10^2$–$13.75*10^2$)	$2*10^2$ ($1*10^2$–$4*10^2$)	$2*10^2$ ($1.50*10^2$–$2.50*10^2$)	0.04*	0.34	0.09
	Enterobacteriaceae	55 (10–90)	5 (0–10)	10 (0–20)	0 (0–10)	0.19	0.21	0.42
Liver	TMABc	$11.50*10^2$ ($4*10^2$–$127*10^2$)	$38.25*10^2$ ($4.38*10^2$–$70.25*10^2$)	$1*10^2$ ($1*10^2$–$2*10^2$)	$1*10^2$ (0–$7*10^2$)	0.05*	0.28	0.95
	Enterobacteriaceae	20 (0–55)	25 (2.5–70)	0 (0–10)	0 (0–20)	0.11	0.69	0.85

[a]High cereal grains group ($n = 9$).
[b]High fibre group ($n = 10$).
*Statistical significance $p < 0.05$.

3.4 | Fatty acid profile of the *Longissimus thoracis et lumborum* muscle

The fatty acid profiles of muscle samples from horses reared using different feeding regimes (HCG vs. HFG) are reported in Table 6. Muscle from horses fed with high amounts of fibre showed an increased concentration of C20:5 ($p = 0.03$), PUFA ($p = 0.05$) and *n*6 ($p = 0.04$) than muscle from horses fed with high amounts of cereal grains.

3.5 | Antioxidant enzymes and oxidation end-products

Table 7 shows the results obtained from oxidative enzyme analyses. Muscular GPx and muscular SOD were higher in samples from HCG compared with those from HFG according to the dietary treatment ($p = 0.01$ and $p = 0.03$), whereas plasma CAT was lower in samples from HCG compared with those from HFG ($p = 0.05$). Of the biochemical metabolites resulting from oxidation pathways (Table 8), higher concentrations of muscular TBARs were evident in samples from HFG compared with samples from HCG ($p = 0.01$).

4 | DISCUSSION

The present study was carried out under field conditions without any possibility of choosing the horses involved in the trial or to change the breeder's management choices for the HCG. As a consequence, it was not possible to establish isoenergetic or isoproteic diets for the two experimental groups. Accordingly, the higher TMABc in the lymph nodes and liver samples found in the HCG could be a consequence of higher bacterial translocation. Regarding the Enterobacteriaceae counts of the liver samples, although no statistically significant difference was detected between groups, it is interesting to note that whilst Enterobacteriaceae were detected in the liver samples from HCG, the median content in HFG was zero.

A multitude of factors may trigger the intestinal barrier dysfunctions that generate a leaky gut, including infectious diseases, drugs, exercise or heat stress (Lambert, 2009). However, in agreement with Stewart et al. (2017), we can hypothesise that the diet was one of the main factors contributing to the differences between the groups of the present study. In fact, all the horses were healthy and admitted to the slaughterhouse without any clinical signs or the requirement for any medical treatment.

Here, we explored selected traits between groups, focusing on the muscle characteristics and chemical composition of the *Longissimus thoracis et lumborum* muscle. In particular, muscle from female horses in HFG showed a higher water holding capacity; and a higher moisture content and a lower pH were identified according to the dietary treatment. In both groups, muscle pH was found to be higher than the values reported in other studies. For example, Gill (2005) reported the pH of horse muscle to be generally below 6.

TABLE 5 Muscle characteristics and chemical composition (HCG vs. HFG)

	HCG[a]		HFG[b]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
pH	6.68 (0.06)	6.70 (0.05)	6.49 (0.07)	6.54 (0.07)	0.02*	0.63	0.85
Water holding capacity (%)	80.27 (0.42)[A]	81.27 (0.81)[AB]	82.37 (0.32)[B]	81.17 (0.08)[AB]	0.04*	0.83	0.03*
Haematin (μg/g)	250.31 (17.88)	236.19 (38.99)	229.87 (26.52)	259.5 (68.66)	0.97	0.83	0.55
L[c]	38.65 (0.58)	39.20 (1.39)	36.23 (0.57)	37.00 (0.28)	0.01*	0.44	0.90
a[d]	16.46 (0.60)	16.65 (0.46)	17.33 (0.22)	16.39 (0.39)	0.50	0.41	0.22
b[e]	−2.46 (0.48)	−1.50 (0.38)	−1.55 (0.18)	−1.04 (0.47)	0.09	0.07	0.56
L*[f]	36.86 (1.29)	37.70 (1.34)	35.98 (0.23)	37.46 (0.28)	0.57	0.24	0.74
a*[g]	15.96 (0.55)	16.75 (0.56)	16.71 (0.35)	16.26 (0.38)	0.71	0.73	0.23
b*[h]	−1.53 (0.34)	−1.01 (0.58)	0.44 (0.17)	−1.20 (0.33)	0.71	0.21	0.88
Moisture (%)	70.44 (0.20)	70.48 (0.51)	71.49 (0.31)	71.63 (0.88)	0.03*	0.84	0.90
Protein (% of DM[i])	75.86 (1.27)	75.34 (2.11)	79.37 (0.82)	80.23 (1.90)	0.01*	0.91	0.64
IMF[j] (% of DM)	11.8 (1.92)	13.08 (2.99)	8.31 (0.85)	7.08 (1.58)	0.03*	0.99	0.52
Ash (% of DM)	4.30 (0.32)	4.83 (0.52)	4.94 (0.29)	4.67 (0.52)	0.56	0.76	0.33

Data shown are means (SEM).

A,B Means with different superscripts differ at $p < 0.05$.

[a]High cereal grains group ($n = 9$).

[b]High fibre group ($n = 10$).

[c]Lightness on fresh samples

[d]Redness on fresh samples.

[e]Yellowness on fresh samples.

[f]Lightness after thawing.

[g]Redness after thawing.

[h]Yellowness after thawing.

[i]Dry matter.

[j]Intramuscular fat.

*Statistical significance $p < 0.05$.

Similarly, Seong et al. (2017) reported pH values around 5.75, with a significant increase in pH the longer samples had been stored (frozen). The low pH values reported in those studies are likely related to the fact that during the development of rigour mortis, muscle glycogen is converted to lactic acid (Lawrie, 1953). After slaughter, glycolysis continues in tissues until the glycogen substrate is depleted, resulting in the accumulation of acidic glycolytic end-products and a drop in pH (Muir et al., 1998). Our results suggest the existence of differences in the biochemical pathways (e.g. the glycolytic rate) underway in the muscle between groups. The high pH values detected in the present study could be due to different levels of muscle glycogen compared to the studies previously cited. Unfortunately, it was not possible to measure the muscular glycogen in this present study.

The values of water holding capacity recorded in this study were in agreement with the data reported in the literature on horse meat (De Palo et al., 2013b; Sarriés & Beriain, 2005). The significantly higher mean value found in the HFG samples vs. those from HCG could be due to the lower fat deposition between muscle fibres, the higher protein content and the higher moisture content (Tateo et al., 2008). A previous study found that increasing the requirements up to 200% in Italian Heavy Draft horses (IHDH) did not affect intramuscular fat content or the water holding capacity of muscle (De Palo, Maggiolino, et al., 2014; De Palo, Tateo, et al., 2014; De Palo et al., 2017), but in those studies, a different breed (IHDH) was studied compared the breed used in our study (Bardigiano). Moreover, the present study revealed a significant effect of feeding regime on both these muscle features. It is likely that the difference in results is due to the different characteristics of the feeding trials, which here focussed on different starch to fibre ratios. In addition, the animals fed HFG were fed less protein and less fat and even the mineral composition was also different.

Even if the diets were not isoenergetic and isoproteic, in the authors' opinion some considerations should be taken into account. Interestingly, no statistical significance between groups was found in slaughter BW and ADG (see Table 3). According to the calculation of the net energy provided to the horses per day, the high cereal grain diet supplied 42.3 MJ more than that provided by the diet characterised by high amounts of fibre. According to the French Institute National de la Recherche Agronomique (INRA), a daily body weight gain of 1 kg/day for a horse weighing 350 kg is possible if the animal

TABLE 6 Fatty acid profile (expressed as % of fatty acid methyl esters) of *Longissimus thoracis et lumborum* muscle samples (HCG vs. HFG). Data shown are means (SEM)

	HCG[a]		HFG[b]		*p*-value		
	Female	Male	Female	Male	Diet	Sex	Diet*Sex
C10:0	0.06 (0.01)	0.05 (0.00)	0.05 (0.01)	0.05 (0.00)	0.25	0.22	0.97
C12:0	0.10 (0.01)	0.11 (0.00)	0.12 (0.01)	0.12 (0.02)	0.24	0.25	0.79
C14:0	2.01 (0.26)	2.30 (0.44)	2.25 (0.44)	1.95 (0.12)	0.70	0.87	0.84
C15:0	0.61 (0.17)	0.53 (0.05)	0.55 (0.06)	0.61 (0.18)	0.76	0.83	0.96
C16:0	28.11(0.69)	27.13 (0.61)	27.05 (0.74)	28.55 (1.40)	0.84	0.77	0.17
C16:1	4.81 (0.27)	4.97 (0.35)	5.00 (0.27)	5.37 (0.37)	0.38	0.42	0.74
C17:0	2.94 (0.48)	3.20 (0.63)	4.45 (1.04)	3.08 (1.87)	0.53	0.62	0.46
C18:0	7.04 (0.45)	6.58 0.41)	6.76 (0.56)	7.14 (0.63)	0.81	0.94	0.48
C18:1	30.42 (0.41)	30.74 (0.47)	29.42 (0.75)	28.85 (2.17)	0.15	0.90	0.65
C20:0	0.12 (0.00)	0.12 (0.01)	0.12 (0.01)	0.15 (0.00)	0.17	0.14	0.15
C18:2n−6	17.23 (0.88)	18.13 (0.77)	17.67 (0.74)	17.16 (1.76)	0.79	0.85	0.49
C18:3n−6	0.02 (0.00)	0.03 (0.00)	0.03 (0.00)	0.03 (0.01)	0.93	0.96	0.62
C18:3n−3	4.55 (0.11)	4.32 (0.24)	4.52 (0.25)	4.84 (0.23)	0.33	0.87	0.28
C20:4n−6	0.64 (0.08)	0.53 (0.03)	0.66 (0.16)	0.74 (0.11)	0.59	0.82	0.32
C20:5n−3	0.02 (0.00)	0.02 (0.00)	0.07 (0.04)	0.03 (0.01)	0.03*	0.46	0.67
C22:0	0.41 (0.01)	0.41 (0.02)	0.45 (0.02)	0.42 (0.02)	0.26	0.58	0.44
C22:6n−3	0.88 (0.07)	0.88 (0.09)	0.87 (0.07)	0.93 (0.55)	0.81	0.73	0.71
SFA[c]	41.47 (0.52)	40.44 (0.71)	41.80 (0.63)	42.06 (0.89)	0.19	0.60	0.38
UFA[d]	58.57 (0.52)	59.60 (0.70)	58.23 (0.63)	57.96 (0.89)	0.18	0.60	0.37
MUFA[e]	35.23 (0.50)	35.71 (0.52)	34.41 (0.87)	34.23 (2.35)	0.30	0.89	0.76
PUFA[f]	22.57 (0.31)	22.89 (0.36)	24.52 (0.68)	24.90 (2.51)	0.05*	0.71	0.98
n3	5.34 (0.17)	4.98 (0.31)	5.50 (0.32)	5.74 (0.28)	0.32	0.65	0.27
n6	17.03 (0.33)	17.91 (0.06)	19.02 (0.48)	19.16 (2.23)	0.04*	0.49	0.62
n6/n3	3.08 (0.12)	3.62 (0.20)	3.51 (0.18)	3.33 (0.23)	0.75	0.40	0.11
SFA/UFA	0.71 (0.02)	0.68 (0.02)	0.72 (0.02)	0.73 (0.02)	0.15	0.62	0.46
SFA/MUFA	1.18 (0.02)	1.13 (0.02)	1.22 (0.04)	1.24 (0.10)	0.14	0.79	0.50
SFA/PUFA	1.79 (0.08)	1.71 (0.10)	1.77 (0.08)	1.79 (0.12)	0.72	0.75	0.62
AI[g]	24.38 (0.81)	24.92 (1.01)	24.87 (0.92)	24.80 (1.90)	0.87	0.84	0.79
TI[h]	2.11 (0.10)	1.89 (0.06)	2.01 (0.11)	2.26 (0.18)	0.27	0.90	0.07

[a]High cereal grains group ($n = 9$).
[b]High fibre group ($n = 10$).
[c]SFA: saturated fatty acids.
[d]UFA: unsaturated fatty acids.
[e]MUFA: monounsaturated fatty acids.
[f]PUFA: polyunsaturated fatty acids.
[g]AI: atherogenic index.
[h]TI: thrombogenic index.
*Statistical significance $p < 0.05$

is supplied with 14 MJ plus its maintenance requirement (46.1 MJ; Martin-Rosset, 2015). These findings indicated that the extra energy level supplied with the high cereal grain diet did not result in a significantly higher daily body weight gain compared with that achieved in the horses of HFG. This finding is surprising since horses in HCG were fed more energy than horses in HFG. Anyways, the high cereal grain diet overcomes the starch digestibility of 2 g of starch/kg BW as suggested by some authors (Durham, 2009; Julliand et al., 2006). Not all the estimated energy of the high cereal grain diet was used because the starch level in the diet exceeded the digestive capacity of the horse's intestine (Durham, 2009). Moreover, an additional point that we should consider is that a high cereal grain diet can cause high glycaemic response, resulting in increased reactivity behaviours (Bulmer et al., 2015; Hothersall & Nicol, 2009). Horses

TABLE 7 Plasma, muscle and hepatic concentrations of glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD)

		HCG[a]		HFG[b]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
GPx[c]	Plasma (μmol/mg) Median (25th–75th)	0.07 (0.07–0.07)	0.09 (0.05–0.16)	0.08 (0.05–0.14)	0.12 (0.07–0.14)	0.40	0.36	0.98
	Muscle (U/mg) Median (25th–75th)	0.14 (0.12–0.23)	0.25 (0.15–0.26)	0.12 (0.11–0.14)	0.13 (0.11–0.14)	0.01*	0.38	0.47
	Liver (μmol/mg) Mean (SEM)	0.26 (0.01)	0.22 (0.02)	0.22 (0.02)	0.24 (0.02)	0.70	0.77	0.11
CAT	Plasma (μmol/mg) Median (25th–75th)	0.84 (0.63–0.88)	1.04 (0.69–1.31)	1.41 (0.83–1.47)	1.20 (1.01–7.15)	0.05*	0.25	0.99
	Muscle (U/mg) Median (25th–75th)	5.03 (3.13–7.04)	3.34 (2.67–4.57)	2.96 (2.78–3.46)	4.03 (2.47–5.52)	0.50	0.60	0.23
	Liver (μmol/mg) Median (25th–75th)	536.89 (517.84–539.79)	519.7 (513.51–537.14)	522.06 (517.88–523.21)	534.79 (516.15–538.61)	0.84	0.92	0.16
SOD	Plasma (μmol/mg) Median (25th–75th)	15.38 (7.88–19.07)	7.80 (4.97–14.73)	6.55 (6.29–17.93)	7.49 (7.02–14.03)	0.71	0.389	0.44
	Muscle (U/mg) Median (25th–75th)	17.60 (14.68–18.26)	16.69 (15.59–18.33)	16.47 (5.48–17.53)	6.56 (5.91–17.54)	0.03*	0.66	0.61
	Liver (μmol/mg) Mean (SEM)	115.86 (1.98)	112.69 (3.90)	111.36 (2.33)	114.82 (1.80)	0.68	0.96	0.26

[a]High cereal grains group ($n = 9$).
[b]High fibre group ($n = 10$).
[c]Expressed as oxidised NADPH content.
*Statistical significance $p < 0.05$.

in HCG may spent more energy in locomotion/reactivity behaviours than horses in HFG. Both considerations should be taken into account for future studies. In conclusion, the extra energy supplied with the high amounts of cereal grains is counterproductive, both from economic and welfare points of view.

Regarding colorimetric patterns, the fresh muscle samples from the HCG group showed higher lightness values compared with those from HFG, whereas these differences did not exist after thawing. Lightness in muscle is related both to the amount of intramuscular fat and to the water content on the cut surface (Mancini & Hunt, 2005). Colour changes in meat from foals are affected by slaughtering age and post-thawing time (De Palo et al., 2012). The different IMF values could explain the tendency towards higher lightness values in muscle from HCG compared with that from HFG, both in fresh and in thawed meat. The significant differences in lightness in fresh muscle could be due to the different water holding capacities, whereas, after thawing and post-thawing water losses, the differences in lightness were not statistically significant. Moreover, muscle colour can also be affected by the fatty acid composition of IMF (Lorenzo et al., 2014); indeed, differences in the fatty acid profiles of the two groups were also revealed here.

The diet is one of the main factors influencing the concentration of IMF in horse muscle (Franco et al., 2013; Lorenzo et al., 2014), and diet can influence the fatty acid profile of IMF (Tateo et al., 2008). In fact, several studies have recently underlined that horse breed, slaughter weight and management practices, including feeding regime, affect the fatty acid composition of horses (Juárez et al., 2009; Lorenzo et al., 2010; Sarriés et al., 2006). However, to the best of our knowledge, no studies have quantified the effects of a feeding regimen based on high amounts of fibre on the fatty acid composition of *Longissimus thoracis et lumborum* muscle of horses. Here, we found that the PUFA concentration was higher in muscle from HFG compared with that from HCG. In particular, this result was related to the higher concentration of n6 PUFAs and n3 eicosapentaenoic acid (EPA, C20:5n–3). These differences likely reflect differences between the two diets supplied. Among raw ingredients of the fibrous pelleted feed oilseeds (flaxseeds and dehulled sunflower seeds) was included at dose of 45 g/day during the final 72 days of the fattening period. Regarding the high cereal grain diet, the fat component was essentially supplied by the maize as a main ingredient. However, the total quantity of fat provided by the two diets was similar (see Table 2; HCG = 285.40 g, HFG = 192.70 g; fat contribution to total energy content: HCG = 8.39%, HFG = 10.14%). Interestingly, although HCG presented a higher IMF concentration, the HFG was characterised by a better fatty acid profile, and this result could provide an important incentive to change the feeding practices of horses reared for meat production (Carrillo et al., 2016).

It has been shown that a higher IMF content results in lower moisture content (Duckett et al., 1993; Reagan et al., 1977). Our data align with the literature since HCG displayed a higher IMF content alongside with lower moisture. The mean moisture content was 70.5% and 71.5% for HCG and HFG muscles samples, respectively, in accordance with previous studies conducted on 11–24 months

TABLE 8 Plasma and muscle concentrations of TBARs, hydroperoxides and carbonylated proteins (HCG vs. HFG)

		HCG[a]		HFG[b]		*p*-value		
		Female	Male	Female	Male	Diet	Sex	Diet*Sex
TBARs[c]	Plasma (nmol MDA/ml) Mean (SEM)	1.33 (0.12)	1.15 (0.12)	1.33 (0.05)	1.17 (0.13)	0.90	0.11	0.94
	Muscle (mg MDA/kg) Mean (SEM)	0.26 (0.02)	0.36 (0.07)	0.50 (0.06)	0.47 (0.07)	0.01*	0.57	0.31
Hydroperoxides	Plasma (μmol/L) Mean (SEM)	5.25 (0.43)	5.40 (0.58)	5.29 (0.22)	5.73 (0.45)	0.66	0.48	0.72
	Muscle μmol/g Median (25th–75th)	0.46 (0.42–0.54)	0.69 (0.48–0.88)	0.55 (0.45–0.59)	0.5 (0.42–0.66)	0.81	0.23	0.11
Carbonylated proteins[d]	Plasma (μmol/ml) Mean (SEM)	98.85 (3.63)	101.10 (10.18)	94.43 (5.71)	90.67 (15.97)	0.41	0.93	0.73
	Muscle (nmol DNPH/mg) Mean (SEM)	1.25 (0.19)	1.43 (0.13)	1.24 (0.12)	1.15 (0.16)	0.39	0.78	0.41

[a]High cereal grains group ($n = 9$).
[b]High fibre group ($n = 10$).
[c]Expressed as malonaldehyde (MDA) content.
[d]Expressed as dinitrophenylhydrazine (DNPH) content.
*Statistical significance $p < 0.05$.

horses (Juárez et al., 2009; Sarriés & Beriain, 2005; Tateo et al., 2008).

Horse muscle is characterised by a high protein content, which varies according to a number of factors, such as sex, muscle type and production system employed (Lorenzo et al., 2014). The French system (Martin-Rosset, 2015) reports that for a daily growth of 1 kg BW, the total dietary protein requirements should be 733 g MADC/day for a horse weighing 350 kg (where MADC—Matières Azotées Digestibles Cheval (MADC)—expresses horse digestible crude protein, which represents the estimated measure of the quality of the absorbed amino acids provided by a diet). According to this, horses in the HFG (with a mean sBW of 344.40 kg) would have needed to consume 692 g MADC/day for an average daily BW gain of 0.96 kg. In this study, the HFG diet provided 723 g MADC/day. On the other hand, horses in the HGC (with a sBW of 347.8 kg) would have needed to consume 735 g MADC/day for a daily BW gain of 1.01 kg. However, the horses in HCG were actually supplied with 1178 g MADC/day.

It is important to note that not only should the protein content of a feed meet the total MADC requirements, but also provide proteins of high biological value. In particular, in horse diets, lysine is the main limiting amino acid, especially if diets are cereal grain-based (Urschel & Lawrence, 2013). In fact, in our study, the horses in HCG received an estimated 48 g of lysine in the diet. On the contrary, the high fibre group was supplied with 76.50 g of lysine. Therefore, these differences could have affected the development of the muscle.

Regarding oxidative status, the higher concentration of PUFAs in muscle samples from HFG compared with that found in HCG could explain the higher muscular concentration of TBARs in the HFG. In fact, the different oxidative stability of IMF is reported to be related to the saturation index of fatty acids (Mahecha et al., 2009). On the contrary, muscular GPx and muscular SOD were higher in HCG than in HFG. Although higher oxidative stress is related to lower GPx and SOD levels, the higher levels in HCG compared with in HFG remains unexplained. In particular, GPx activities are related to selenium intake, and a low selenium intake is related to low GPx activities and vice versa (Avellini et al., 1999). As shown in Table 2, the horses in HCG received only 400 mg of Vitamin E and 0.48 mg of selenium per day, whereas those in HFG were supplied with 1105 mg of Vitamin E and 1.72 mg of selenium. Selenium and Vitamin E are dietary antioxidants which synergistically support endogenous antioxidant systems to reduce reactive oxygen species damages. Limited data are reported from experimental feeding trials on effective nutritional supplementation in Vitamin E in horse meat. However, taking into account scientific studies carried out on other species (Cardenia et al., 2011; Voljč et al., 2011), the α-tocopherol levels—natural isoform of the fat-soluble vitamin E group—in tissues and plasma were significantly influenced by the level of dietary supplementation, leading to higher stability of meat lipids. Moreover, Cappai, Pudda et al., (2020); Cappai, Taras et al., (2020) recommended to monitor the Vitamin E intake in the context of adequate feeding practices for health and welfare assessment. In particular, since α-tocopherol is synthesised and stored chiefly in the green plants, the same authors

suggested that a higher dietary intake of Vitamin E is important in stabled horses when they are fed on hay.

Finally, the higher plasma levels of CAT in the horses belonging to HFG suggest that the animals tended to be protected from oxidative damage, as this enzyme is involved in one of the most rapid and effective systems for reducing oxygen free radicals (Ighodaro & Akinloye, 2018). A high fibre source in the diet can effectively promote antioxidant defence by enhancing the free radical-scavenging ability of the plasma and other relevant organs (Fang et al., 2017). However, no studies have been carried out to date on the antioxidative effects of dietary fibre intake and different fibre components on horse tissue. Even if in this study group replication was not possible, and it is certainly important, this does not preclude the fact that this study can be a source of important suggestions for further studies.

5 | CONCLUSIONS

The present study shows that feeding horses high amounts of cereal grains is wasteful from an economic stance and harmful from a welfare point of view. In fact, the high amounts cereal grains in the diet did not result with any difference in daily bodyweight gain or with any positive effect on muscle characteristics. Instead, our results support the notion that feeding horses high amounts of cereal grains can lead to a condition of increased intestinal permeability. We also showed that diet affects the concentrations of GPx, CAT and SOD; although plasma, muscle and liver were characterised by distinct differences. We hope this work will encourage further scientific research to improve the feeding practices used in horses' farms in order to safeguard the welfare of horses reared for meat purposes encouraging adequate education of farmers.

6 | ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation.

ACKNOWLEDGEMENTS

The authors would like to thank Acme S.r.l. for providing the first month's free supply of fibrous pelleted feed for this trial. The authors also thank the farmer and the slaughterhouse staff who allowed us to carry out the sampling. The authors Emanuela Valle and Federica Raspa highlight that the present study was not performed to promote horse meat consumption; instead, its aim was to improve the quality of life of this category of horse, taking into account the farming management conditions these animals are subjected to. In fact, welfare of horses reared for meat production is often overlooked and assessment for setting standards at farm level appears poor. Open Access Funding provided by Universita degli Studi di Torino within the CRUI-CARE Agreement. WOA Institution: Universita degli Studi di Torino Blended DEAL: CARE

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Federica Raspa https://orcid.org/0000-0002-9298-3045
Francesca Rita Dinardo https://orcid.org/0000-0002-1109-4431
Ingrid Vervuert https://orcid.org/0000-0001-7151-0751
Domenico Bergero https://orcid.org/0000-0001-5525-1534
Maria Teresa Bottero https://orcid.org/0000-0002-0803-9730
Daniele Pattono https://orcid.org/0000-0002-0813-5810
Alessandra Dalmasso https://orcid.org/0000-0002-9991-9046
Pasquale De Palo https://orcid.org/0000-0002-5612-1691
Emanuela Valle https://orcid.org/0000-0002-5519-3554

REFERENCES

Avellini, L., Chiaradia, E., & Gaiti, A. (1999). Effect of exercise training, selenium and vitamin E on some free radical scavengers in horses (*Equus caballus*). *Comparative Biochemistry and Physiology*, *123*, 147–154. https://doi.org/10.1016/S0305-0491(99)00045-0

Bulmer, L., McBride, S., Williams, K., & Murray, J. A. (2015). The effects of a high-starch or high-fibre diet on equine reactivity and handling behaviour. *Applied Animal Behaviour Science*, *165*, 95–102. https://doi.org/10.1016/j.applanim.2015.01.008

Cappai, M. G., Alesso, G. A., Nieddu, G., Sanna, M., & Pinna, W. (2013). Electron microscopy and composition of raw acorn starch in relation to in vivo starch digestibility. *Food and Function*, *4*, 917–922. https://doi.org/10.1039/c3fo60075k

Cappai, M. G., Pudda, F., Wolf, P., Accioni, F., Boatto, G., & Pinna, W. (2020). Variation of hematochemical profile and vitamin E status in Feral Giara Horses from free grazing in the wild to hay feeding during captivity. *Journal of Equine Veterinary Science*, *94*(2), 103220. https://doi.org/10.1016/j.jevs.2020.103220

Cappai, M. G., Taras, A., Cossu, I., Cherchi, R., Dimauro, C., Accioni, F., Boatto, G., Deroma, M., Spanu, E., Gatta, D., Dall'Aglio, C., & Pinna, W. (2020). Effects of dietary Zn/Se and α-tocopherol supplementation on metabolic milieu, haemogram and semen traits of breeding stallions. *Biological Trace Element Research*, *199*(9), 3287–3296. https://doi.org/10.1007/s12011-020-02447-7

Cardenia, V., Rodriguez-Estrada, M. T., Cumella, F., Sardi, L., Della Casa, G., & Lercker, G. (2011). Oxidative stability of pork meat lipids as related to high-oleic sunflower oil and vitamin E diet supplementation and storage conditions. *Meat Science*, *88*, 271–279. https://doi.org/10.1016/j.meatsci.2010.12.034

Carrillo, J. A., He, Y., Li, Y., Liu, J., Erdman, R. A., Sonstegard, T. S., & Song, J. (2016). Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare. *Scientific Reports*, *6*, 25948. https://doi.org/10.1038/srep25948

Celi, P., & Gabai, G. (2015). Oxidant/antioxidant balance in animal nutrition and health: The role of protein oxidation. *Frontiers in Veterinary Medicine*, *2*, 48. https://doi.org/10.3389/fvets.2015.00048

Davidson, N., & Harris, P. (2007). Nutrition and welfare. In *The welfare of horses* (pp. 45–76). Springer. https://doi.org/10.1007/978-1-4020-3227-1

Davis, J. L., Blikslager, A. T., Catto, K., & Jones, S. L. (2003). A retrospective analysis of hepatic injury in horses with proximal enteritis

(1984–2002). *Journal of Veterinary Internal Medicine*, *17*(6), 896–901. https://doi.org/10.1892/0891-6640(2003)017<0896:ARAOHI>2.3.CO;2

De Palo, P., Maggiolino, A., Albenzio, M., Casalino, E., Neglia, G., Centoducati, G., & Tateo, A. (2018). Survey of biochemical and oxidative profile in donkey foals suckled with one natural and one semi-artificial technique. *PLoS One*, *13*(6), e0198774. https://doi.org/10.1371/journal.pone.0198774

De Palo, P., Maggiolino, A., Centoducati, N., & Tateo, A. (2015). Effects of different milk replacers on carcass traits, meat quality, meat color and fatty acids profile of dairy goat kids. *Small Ruminant Research*, *131*, 6–11. https://doi.org/10.1016/j.smallrumres.2015.09.001

De Palo, P., Maggiolino, A., Centoducati, P., Milella, P., Calzaretti, G., & Tateo, A. (2016). Is meat quality from *Longissimus lumborum* samples correlated with other cuts in horse meat? *Animal Science Journal*, *87*(3), 428–438. https://doi.org/10.1111/asj.12433

De Palo, P., Maggiolino, A., Centoducati, P., & Tateo, A. (2012). Colour changes in meat of foals as affected by slaughtering age and post-thawing time. *Asian-Australasian Journal of Animal Sciences*, *25*(12), 1775–1779. https://doi.org/10.5713/ajas.2012.12361

De Palo, P., Maggiolino, A., Centoducati, P., & Tateo, A. (2013a). Effects of two different packaging materials on veal calf meat quality and shelf life. *Journal of Animal Science*, *91*(6), 2920–2930. https://doi.org/10.2527/jas.2012-5292

De Palo, P., Maggiolino, A., Centoducati, P., & Tateo, A. (2013b). Slaughtering age effect on carcass traits and meat quality of italian heavy draught horse foals. *Asian-Australasian Journal of Animal Sciences*, *26*(11), 1637–1643. https://doi.org/10.5713/ajas.2013.13174

De Palo, P., Maggiolino, A., Tateo, A., & Centoducati, P. (2014). Influence of gas mixture on quality and shelf life of veal calf meat. *Italian Journal of Animal Science*, *13*(3129), 226–233. https://doi.org/10.4081/ijas.2014.3129

De Palo, P., Tateo, A., Maggiolino, A., & Centoducati, P. (2014). Effect of nutritive level on carcass traits and meat quality of IHDH foals. *Animal Science Journal*, *85*(7), 780–786. https://doi.org/10.1111/asj.12203.

De Palo, P., Tateo, A., Maggiolino, A., Marino, R., Ceci, E., Nisi, A., & Lorenzo, J. M. (2017). Martina Franca donkey meat quality: Influence of slaughter age and suckling technique. *Meat Science*, *134*, 128–134. https://doi.org/10.1016/j.meatsci.2017.07.025

Duckett, S. K., Wagner, D. G., Yates, L. D., Dolezal, H. G., & May, S. G. (1993). Effects of time on feed on beef nutrient composition. *Journal of Animal Science*, *71*, 2079–2088. https://academic.oup.com/jas/article/71/8/2079/4718971. https://doi.org/10.2527/1993.7182079x

Durham, A. E. (2009). The role of nutrition in colic. *Veterinary Clinics of North America: Equine Practice*, *25*, 67–78. https://doi.org/10.1016/j.cveq.2008.11.003

Fang, T., Wu, X., Cao, W., Jia, G., Zhao, H., Chen, X., Wu, C., Tang, J., Wang, J., & Liu, G. (2017). Effects of dietary fiber on the antioxidant capacity, immune status, and antioxidant-relative signaling molecular gene expression in rat organs. *RSC Advances*, *7*(32), 19611–19620. https://doi.org/10.1039/c7ra02464a

Faure, P., & Lafond, J. L. (1995). Measurement of plasma sulfhydryl and carbonyl groups as a possible indicator of protein oxidation. In *Analysis of Free Radicals in Biological Systems* (pp. 237–248). https://doi.org/10.1007/978-3-0348-9074-8_17

Franco, D., Crecente, S., Antonio Vázquez, J., Gómez, M., & Lorenzo, J. M. (2013). Effect of cross breeding and amount of finishing diet on growth parameters, carcass and meat composition of foals slaughtered at 15 months of age. *Meat Science*, *93*, 547–556. https://doi.org/10.1016/j.meatsci.2012.11.018

Geor, R. J., & Harris, P. A. (2007). How to minimize gastrointestinal disease associated with carbohydrate nutrition in horses. *Proceedings of the Annual Convention of the AAEP*, *53*, 178–185.

Gill, C. O. (2005). Safety and storage stability of horse meat for human consumption. *Meat Science*, *71*, 506–513. https://doi.org/10.1016/j.meatsci.2005.04.030

Gondim, F. J., Zoppi, C. C., dos Reis Silveira, L., Pereira-da-Silva, L., & Vaz de Macedo, D. (2009). Possible relationship between performance and oxidative stress in endurance horses. *Journal of Equine Veterinary Science*, *29*(4), 206–212. https://doi.org/10.1016/j.jevs.2009.02.006

Hornsey, H. C. (1956). The colour of cooked cured pork. I.—Estimation of the nitric oxide-haem pigments. *Journal of the Science of Food and Agriculture*, *7*(8), 534–540. https://doi.org/10.1002/jsfa.2740070804

Hothersall, B., & Nicol, C. (2009). Role of diet and feeding in normal and stereotypic behaviors in horses. *Veterinary Clinics of North America: Equine Practice*, *25*(1), 167–181. https://doi.org/10.1016/J.CVEQ.2009.01.002

Hudson, J. M., Cohen, N. D., Gibbs, P. G., & Thompson, J. A. (2001). Feeding practices associated with colic in horses. *Journal of the American Veterinary Medical Association*, *219*(10), 1419–1425. https://doi.org/10.2460/javma.2001.219.1419

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001

Juárez, M., Polvillo, O., Gómez, M. D., Alcalde, M. J., Romero, F., & Valera, M. (2009). Breed effect on carcass and meat quality of foals slaughtered at 24 months of age. *Meat Science*, *83*, 224–228. https://doi.org/10.1016/j.meatsci.2009.04.022

Julliand, V., De Fombelle, A., & Varloud, M. (2006). Starch digestion in horses: The impact of feed processing. *Livestock Science*, *100*(1), 44–52. https://doi.org/10.1016/j.livprodsci.2005.11.001

Lambert, G. P. (2009). Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *Journal of Animal Science*, *87*(14 Suppl), 101–108. https://doi.org/10.2527/jas.2008-1339

Lawrie, R. A. (1953). The onset of rigor mortis in various muscles of the draught horse. *Journal of Physiology*, *I953*(121), 275–288. https://doi.org/10.1113/jphysiol.1953.sp004947

Lesimple, C. (2020). Indicators of horse welfare: State-of-the-Art. *Animals*, *10*(2), 294. https://doi.org/10.3390/ani10020294

Lorenzo, J. M., Fuciños, C., Purriños, L., & Franco, D. (2010). Intramuscular fatty acid composition of "Galician Mountain" foals breed effect of sex, slaughtered age and livestock production system. *Meat Science*, *86*, 825–831. https://doi.org/10.1016/j.meatsci.2010.07.004

Lorenzo, J. M., Sarriés, M. V., Tateo, A., Polidori, P., Franco, D., & Lanza, M. (2014). Carcass characteristics, meat quality and nutritional value of horsemeat: A review. *Meat Science*, *96*(4), 1478–1488. https://doi.org/10.1016/j.meatsci.2013.12.006

MacLeay, J. M., Sorum, S. A., Valberg, S. J., Marsh, W. E., & Sorum, M. D. (1999). Epidemiologic analysis of factors influencing exertional rhabdomyolysis in thoroughbreds. *American Journal of Veterinary Research*, *60*(12), 1562–1566. https://europepmc.org/article/med/10622169

Maggiolino, A., Lorenzo, J. M., Salzano, A., Faccia, M., Blando, F., Serrano, M. P., Latorre, M. A., QuinÕnes, J., & De Palo, P. (2020). Effects of aging and dietary supplementation with polyphenols from Pinus taeda hydrolysed lignin on quality parameters, fatty acid profile and oxidative stability of beef. *Animal Production Science*, *60*, 713–724. https://doi.org/10.1071/AN19215

Mahecha, L., Nuernberg, K., Nuernberg, G., Ender, K., Hagemann, E., & Dannenberger, D. (2009). Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls. *Meat Science*, *82*(3), 365–371. https://doi.org/10.1016/j.meatsci.2009.02.005

Mainar-Jaime, R. C., Andrés, S., Vico, J. P., San Román, B., Garrido, V., & Grilló, M. J. (2013). Sensitivity of the ISO 6579:2002/Amd 1:2007

standard method for detection of *Salmonella* spp. on mesenteric lymph nodes from slaughter pigs. *Journal of Clinical Microbiology*, *51*(1), 89–94. https://doi.org/10.1128/JCM.02099-12

Mancini, R. A., & Hunt, M. C. (2005). Current research in meat color. *Meat Science*, *71*, 100–121. https://doi.org/10.1016/j.meatsci.2005.03.003

Martin-Rosset, W. (2015). The growing horse. In *Equine Nutrition: INRA nutrient requirements, recommended allowances and feed tables* (pp. 169–215). Academic Publishers.

Merritt, A. M., & Julliand, V. (2013). Gastrointestinal physiology. In *Equine applied and clinical nutrition* (pp. 3–32). E-Book: Health, Welfare and Performance; Elsevier Ltd. https://doi.org/10.1016/B978-0-7020-3422-0.00001-8

Muir, P. D., Deaker, J. M., & Bown, M. D. (1998). Effects of forage- and grain-based feeding systems on beef quality: A review. *New Zealand Journal of Agricultural Research*, *41*, 623–635. https://doi.org/10.1080/00288233.1998.9513346

Raspa, F., Tarantola, M., Bergero, D., Bellino, C., Mastrazzo, C. M., Visconti, A., Valvassori, E., Vervuert, I., & Valle, E. (2020). Stocking density affects welfare indicators in horses reared for meat production. *Animals*, *10*(6), 1103. https://doi.org/10.3390/ani10061103

Raspa, F., Tarantola, M., Bergero, D., Nery, J., Visconti, A., Mastrazzo, C. M., Cavallini, D., Valvassori, E., & Valle, E. (2020). Time-budget of horses reared for meat production : influence of stocking density on behavioural activities and subsequent welfare. *Animals*, *10*, 1334. https://doi.org/10.3390/ani10081334

Reagan, J. O., Carpenter, J. A., Bauer, F. T., & Lowrey, R. S. (1977). Packaging and palatability characteristics of grass and grass-grain beef. *Journal of Animal Science*, *46*(4), 716–721.

Sarriés, M. V., & Beriain, M. J. (2005). Carcass characteristics and meat quality of male and female foals. *Meat Science*, *70*, 141–152. https://doi.org/10.1016/j.meatsci.2004.12.006

Sarriés, M. V., Murray, B. E., Troy, D., & Beriain, M. J. (2006). Intramuscular and subcutaneous lipid fatty acid profile composition in male and female foals. *Meat Science*, *72*, 475–485. https://doi.org/10.1016/j.meatsci.2005.08.014

Seong, P. N., Seo, H. W., Kim, J. H., Kang, G. H., Cho, S. H., Chae, H. S., Park, B. Y., & Van Ba, H. (2017). Assessment of frozen storage duration effect on quality characteristics of various horse muscles. *Asian-Australasian Journal of Animal Sciences*, *30*, 1756. –1763. https://doi.org/10.5713/ajas.17.0039

Södergren, E., Nourooz-Zadeh, J., Berglund, L., & Vessby, B. (1998). Re-evaluation of the ferrous oxidation in xylenol orange assay for the measurement of plasma lipid hydroperoxides. *Journal of Biochemical and Biophysical Methods*, *37*(3), 137–146. https://doi.org/10.1016/S0165-022X(98)00025-6

Stewart, A. S., Pratt-Phillips, S., & Gonzalez, L. M. (2017). Alterations in intestinal permeability: the role of the "Leaky Gut" in health and disease. *Journal of Equine Veterinary Science*, *52*, 10–22. https://doi.org/10.1016/j.jevs.2017.02.009

Tateo, A., De Palo, P., Ceci, E., & Centoducati, P. (2008). Physicochemical properties of meat of Italian Heavy Draft horses slaughtered at the age of eleven months. *Journal of Animal Science*, *86*(5), 1205–1214. https://doi.org/10.2527/jas.2007-0629

Tateo, A., Maggiolino, A., Domínguez, R., Lorenzo, J. M., Dinardo, F. R., Ceci, E., Marino, R., Della Malva, A., Bragaglio, A., & De Palo, P. (2020). Volatile organic compounds, oxidative and sensory patterns of vacuum aged foal meat. *Animals*, *10*(9), 1–17. https://doi.org/10.3390/ani10091495

Tufarelli, V., Laudadio, V., & Casalino, E. (2016). An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in meat-type broiler chickens. *Environmental Science and Pollution Research*, *23*, 6197–6204. https://doi.org/10.1007/s11356-015-5852-1

Urschel, K. L., & Lawrence, L. M. (2013). Amino acids and protein. In *Equine applied and clinical nutrition: Health, welfare and performance* (pp. 113–135). Saunders Elsevier. https://doi.org/10.1016/B978-0-7020-3422-0.00006-7

Valberg, S. J., Macleay, J. M., Billstrom, J. A., Hower-Moritz, M. A., & Mickelson, J. R. (1999). Skeletal muscle metabolic response to exercise in horses with 'tying-up' due to polysaccharide storage myopathy. *Equine Veterinary Journal*, *31*(1), 43–47. https://doi.org/10.1111/j.2042-3306.1999.tb03789.x

Voljč, M., Frankič, T., Levart, A., Nemec, M., & Salobir, J. (2011). Evaluation of different vitamin e recommendations and bioactivity of α-tocopherol isomers in broiler nutrition by measuring oxidative stress in vivo and the oxidative stability of meat. *Poultry Science*, *90*(7), 1478–1488. https://doi.org/10.3382/ps.2010-01223

Webb, H. E., Brichta-Harhay, D. M., Brashears, M. M., Nightingale, K. K., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Schmidt, J. W., Wang, R., Granier, S. A., Brown, T. R., Edrington, T. S., Shackelford, S. D., Wheeler, T. L., & Loneragan, G. H. (2017). Salmonella in peripheral lymph nodes of healthy cattle at Slaughter. *Frontiers in Microbiology*, *8*(224), 1–10. https://doi.org/10.3389/fmicb.2017.02214

Williamson, A., Rogers, C., & Firth, E. (2011). A survey of feeding, management and faecal pH of Thoroughbred racehorses in the North Island of New Zealand. *New Zealand Veterinary Journal*, *55*(6), 337–341. https://doi.org/10.1080/00480169.2007.36790

How to cite this article: Raspa, F., Dinardo, F. R., Vervuert, I., Bergero, D., Bottero, M. T., Pattono, D., Dalmasso, A., Vinassa, M., Valvassori, E., Bruno, E., De Palo, P., & Valle, E. (2021). A Fibre- vs. cereal grain-based diet: Which is better for horse welfare? Effects on intestinal permeability, muscle characteristics and oxidative status in horses reared for meat production. *Journal of Animal Physiology and Animal Nutrition*, 00, 1–14. https://doi.org/10.1111/jpn.13643