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DIETARY SUPPLEMENTATION OF A SINGLE HERB (*Silybum marianum*) AND A MIX OF SELECTED HERBS AND SPICES IN GROWING RABBITS

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1. INTRODUCTION

The health status influences the growing phase of the rabbits. Particularly the pre- and post-weaning period is the most critical phase: milk is substituted with solid feed, the kits' immune system is still immature and the kits are separated from their mothers (Carabaño *et al*., 2006; Gidenne *et al*., 2005). Digestive disturbances are the main cause of the morbidity and mortality that create important economic losses for rabbit farmers (Marlier *et al*., 2006; Licois, 2004). For this reason some antibiotic growth promoters have been practiced in the United States and some other countries, but their usefulness was contested, since some similar antibiotics are used in human medicine and their use contribute to the pool of antibiotic resistant bacteria. Thus, in 2006 the use of antibiotics as growth promoters for farmed animals has been banned in the EU due to safety issues, health concerns as well as increasing demand of consumers for more natural products (Barug *et al*., 2006 Falcão-e-Cunha *et al.,* 2007). Therefore, in order to keep ensuring satisfactory performances as well as low morbidity and mortality of farmed animals, other potential substitutes of natural origin were contemplated to improve health status and productive performance of the animal. These natural additives were divided on: probiotics (live microrganisms that confer a health effect on the host when consumed in adequate amounts (Guaerner & Schaafsma, 1998), prebiotics: food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon and to improve host health (Gibson & Robertfroid (1995); enzymes: the commercial use of enzymes has started less than 20 years ago(Choct, 2006); organics

acids: they have been used in the feed industry, recently recognized to possess favourable effects on growing rabbits (Skřivanová & Marounek, 2002 Romero *et al*. 2011).

Herbs, spices, and botanicals are classified by habitat, part used, therapeutic value, and type of administration (Dalle Zotte *et al*., 2016). Since the beginning of the history, humans used plants and spices for their nutritional and medicinal properties. Although the distinction between herbs and spices is blurred, it has been suggested that herbs tend to be of leaf origin and spices of stem, bark, and seed origin. Vaunting a wide range of activities, some have been associated with improvements in animal performance and increased nutrient availability. Plants have developed a range of low molecular weight secondary metabolites, called phytochemicals, that help to prevent physiological and environmental stress and oppose pathogens (Wenk, 2003). Most of these active secondary metabolites are in the class of isoprene derivatives, flavonoides and glucosinolates.

These natural additives have received closer attention from the feed industry in recent years. Many studies have described herbal plants as additives in rabbit feeding, but the *in vivo* studies are still limited (Dalle Zotte and Szendrő, 2011; Dalle Zotte *et al*., 2016). Moreover, some plant extract showed to possess a certainly toxic effect (Samson *et al*., 2012).

The utilization of herbs and spices in animal nutrition focuses on the potential benefic effect given by the phyotochemical compounds on the digestive system, as antimicrobial, antioxidant and as a growth promoter. Phenolic compounds are the largest group of secondary metabolites identified in plants; they include simple phenols, flavonoids, lignins and lignans, tannins, xanthones and coumarins (Huang *et al*., 2010). Different authors showed positive effect in productive performances, where the

plants or a mixture of them had the ability to influence the digestive system, reducing the mortality and improving growth performances (Omer *et al*., 2012; Omer *et al*., 2013; Matusevicius *et al*., 2011; Rotolo *et al*., 2013). Antimicrobial effect is considered peculiar effect of plant essential oil, with thymol and carvacrol as examples of active components (Helander *et al*., 1998 Lambert *et al*., 2001). The dietary supplementation of a mix of plants (Digestarom®) or a single plant (*Silybum marianum*) to growing rabbits reduced mortality but the impact on digestive diseases is still controversial (Krieg *et al.*, 2009; Kosina *et al*., 2017).

Phenolic substances present in plants and plant products are also capable of oxidative action. They are used for multiple purposes as protecting animal feeds during storage, supporting the defence of the tissues in the alive animals, and diminishing oxidative reaction in meat and meat products (Vekiari *et al*., 1993; McCarthy *et al*., 2001; Botsoglou *et al*., 2004; Kulisic *et al*., 2004; Shan *et al*., 2005; Collin, 2006; Coma, 2008; Soultos *et al*., 2009; Zinoviadou *et al*., 2009; Eid *et al*., 2011; Dal Bosco *et al*., 2014; Dalle Zotte *et al*., 2014; Cardinali *et al*., 2015).

In the next chapter is presented a detailed overview of the literature focusing on the dietary use of herbs and spices in the growing rabbit and meat quality.

2. REVIEW OF LITERATURE

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Review article

Herbs and spices inclusion as feedstuff or additive in growing rabbit diets and as additive in rabbit meat: A review

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ABSTRACT

The European ban on the non-therapeutic use of antibiotic growth promoters and limits on the use of other drugs have increased digestive disorders and mortality in growing rabbits. In addition, consumers demand natural products, and therefore synthetic active compounds should be replaced by natural ones. This has increased the search for alternatives, such as herbs, spices and their extracts (botanicals) as replacers. Plants (whole plants, leaves or seeds, mainly used as feedstuffs) and their extracts (considered as additives) are being increasingly used in animal nutrition as appetisers, digestive and physiological stimulants, colorants, and antioxidants, and for the prevention and treatment of certain pathological conditions. The digestive effects of herbs and spices have been tested primarily in humans and laboratory animals, and few trials have been performed on farm animals. Studies on the dietary inclusion of herbs and spices or their extracts in rabbit meat production are quite scarce, and the overall benefit remains unclear due to discrepancies in results, such as the use of plant preparations as galactagogues in rabbit does. Some positive results have been shown their potential, however. The dietary inclusion of *Foeniculum vulgare* Mill. seeds with oregano leaves has been observed to improve diet utilisation, whereas the dietary inclusion of a mixture of *Lupinus albus* L., *Trigonella foenum-graecum* L., and *Cassia senna* L. has acted as growth promoter. Antimicrobial effects are derived especially from plant volatile oils. In the rabbit, a stabilizing effect on microbiota was observed when the diet was supplemented with thyme oil. When diets were supplemented with thyme leaves and spirulina algae, an antimicrobial effect on *Clostridium coccoides, Clostridium leptum* in the caecum was observed. Black cumin seeds have been shown to exert anti-inflammatory, anti-bacterial and immunomodulatory effects. Several herbs and spices (green tea, rooibos, oregano, rosemary and thyme) provide antioxidant effects through rabbit dietary supplementation or inclusion in meat and meat products. Research in the use of herbs or/and spices has demonstrated their potential as feed additives and/or antioxidants, but further research is recommended to optimize effects on rabbits before practical proposals can be drafted.

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1. Introduction

In growing rabbits, particularly weaners, digestive disturbances are the main cause of the morbidity and mortality that create important economic losses for rabbit farmers (Marlier et al., 2006; Licois, 2004). Weaning is the period in which the kits are separated from their mothers, milk is substituted with solid feed, and the kits' immune system is still immature (Carabaño et al., 2006; Gidenne et al., 2005).

Digestive disturbances may originate from infection, bacteria (enteropathogenic *Escherichia coli* (EPEC), and *Clostridium spp.*) or parasites (Coccidia), or may be included under the term "non specific enteritis", in which feeding and animal stress seem to be the most likely triggering agents that provoke different and atypical clinical symptoms, intestinal lesions and diarrhoea, in particular.

The gastrointestinal syndrome known as Epizootic Rabbit Enteropathy (ERE) characterized by aqueous diarrhoea, abdominal bloating, and the distension of the stomach or the small intestine, has been observed in Europe since 1997. Although ERE is responsible for very high morbidity and mortality rates (up to 70% in growing rabbits), the aetiology of this intestinal disease remains difficult to establish. Some authors (Marlier et al., 2006; Szalo et al., 2007; Marlier, 2015) have postulated that the presence of *Clostridium perfringens* may be involved.

The European ban on AGPs in animal feeds and restrictions in the use of other drugs began in 1986 (Barug et al., 2006). As a consequence of the ban, researchers and feed companies have increased their efforts to develop safer and more natural feed additives, improving both the intestinal health and productivity of broiler rabbits in the meantime.

Researchers must now face the challenge of meeting the requests of increasingly informed and demanding consumers for products that provide similar effects of natural and controlled origin, the so-called pronutrients (Rosen, 1996). These natural additives can be divided into probiotics (Guarner and Schaafsma, 1998), prebiotics (Gibson and Roberfroid, 1995), enzymes (García-Ruiz et al., 2006; Choct, 2006) and organic acids (Skřivanová and Marounek, 2002; Romero et al., 2011).

Herbs, spices, and their extracts (botanicals) are classified by habitat, part used, therapeutic value, and type of administration. Although the distinction between herbs and spices is blurred, it has been suggested that herbs tend to be of leaf origin and spices of stem, bark, and seed origin (Collin, 2006). They cover a wide range of activities and some have been associated with improvements in animal performance and increased nutrient availability. Plants have developed a range of low molecular weight secondary metabolites that help them to prevent physiological and environmental stress, and oppose pathogens (Wenk, 2003a). Most of these active secondary metabolites are isoprene derivatives, flavonoides and glucosinolates. Reports on the effects of this category of feed additives on rabbit growth performance (Omer et al., 2012), antioxidant, and antibacterial activity (Al-Turki, 2007), meat quality (Cardinali et al., 2012), blood biochemical parameters (Al-Jowari, 2012), reproductive performance (El-Nattat and El-Kady, 2007) and doe milk production (Eiben et al., 2004) are still fairly scarce, however.

2. Herbs and spices as feed additives

Worldwide interest in herbal products has grown significantly. As described by Viegi et al. (2003) cattle, horses, sheep, goats and pigs represent about 31%, 14%, 17%, 17% and 7%, respectively, of the animals treated with herbal remedies, followed by poultry (9.1%), dogs (5.3%) and rabbits (4.3%). This is not only due to a general trend toward the use of natural products for curing illnesses but also the availability of mounting evidence regarding the efficacy of herbal remedies.

Herbs, spices and botanicals have been shown to offer a wide range of activities, including animal performance and increasing nutrient availability. When compared to antibiotics or inorganic chemicals, they present less toxicity and are free of unwanted residues, and also act as growth promoters when used as supplements in animal diets, rabbit feed included (Falcao-E-Cunha et al., 2007).

Plants and their extracts are therefore being increasingly used in animal nutrition as appetisers, digestive stimulants, stimulants of physiological functions, colorants, and antioxidants, as well as for the prevention and treatment of certain pathological conditions.

2.1. Plant secondary compounds and biological plausibility

Plants produce chemical compounds as part of their normal metabolic activities. They can be divided into primary (sugar and oils) and secondary compounds (phytochemicals). These organic chemical compounds may affect animal health when administered.

Phytochemicals can be classified by their therapeutic values (antibacterial, antifungal, anti-inflammatory, antiulcer, antioxidant, antiviral, anticancer, or immune stimulants) and preparation modes (tincture, decoction, maceration, syrup, inhalation and infusions). The sub-classes that comprise the phytochemicals are mainly herbs, valued for their medicinal properties, flavour or scent. As noted above, herbs are flowering plants whose stem does not become woody and persistent. Spices are defined as any of a class of pungent or aromatic substances of vegetable origin such as pepper (*Piperaceae* Family), cinnamon (*Cinnamomum zeylonicum*), and cloves (*Syzygium aromaticum*) used as seasonings, preservatives *etc.* A botanical is a drug (extract) made from a part of a plant (roots, stem, bark, leaves, seeds, flowers, fruits). Fungi, algae, and lichens are also considered botanicals. Depending on the extraction method, botanicals can be found as essential oils (steam distilled) that are highly concentrated and volatile, or botanical oils (cold pressed or extracted by heat) that are fatty and non-volatile (Wenk, 2003b; Hashemi and Davoodi, 2011).

Plant extracts or essential oils have distinct odours and are used mainly in the production of perfumes, flavours and pharmaceuticals. They are a rich source of biologically active compounds and have been recognized as having antifungal (Daouk et al., 1995), antioxidant (Burits and Bucar, 2000), and antimicrobial (Cox et al., 2000; Soultos et al., 2009) actions. Most active phytochemicals are believed to act as antibiotics or antioxidants both *in vivo* and in food (Wenk, 2003a). Several authors have dedicated attention to physiologically active secondary plant metabolites (Rhodes, 1996) and the mechanisms of their antioxidant features (Halliwell et al., 1995).

3. Herbs and spices in animal feeding

Herbal plants could be considered a new class of growth promoters, and these feed additives have received closer attention from the feed industry in recent years. Although many studies have described herbal plants as additives in rabbit feeding, most of these works have focused on the use of phytogenics as dietary supplementation in essential oil form, whereas the *in vivo* studies for rabbit species are quite limited (see reviews of Falcao-E-Cunha et al. (2007) and Dalle Zotte and Szendrő (2011)). Their effects on animal health, performance, and meat quality are described below by function.

3.1. Digestive function effect

Although herbs and spices can regulate feed intake and stimulate digestive secretions, they affect digestive processes differently due to the wide variety of phytochemicals. Turmeric (*Curcuma longa*), ginger (*Zingiber officinale*), anise (*Pimpinella anisum*), cayenne pepper, mint (*Mentha* genus), onion, cumin (*Cuminum cyminum* L.), and fenugreek have been shown to enhance the synthesis of bile acids with beneficial effects on digestion and lipid absorption (Frankič et al., 2009). Most of the spices above also stimulate the secretion of pancreatic enzymes (lipases, amylases and proteases) and/or increase the activity of digestive enzymes of gastric mucosa (Srinivasan, 2005). Some, such as red pepper, act as digestive stimulants by enhancing the secretion of saliva and of salivary amylose activity, thus stimulating gastric secretions in humans (Blumenthal et al., 1998). Spices known for their appetite stimulant effect are cinnamon, caraway (*Carum carvi* L.), cloves, cardamom (*Zingiberaceae* Family), bay laurel (*Laurus nobilis* L.), mint, gentian (Baytop, 1984; Wichtl, 1994), ginger and *Carum ajowan* beverages (Wadikar and Premavalli, 2011). Water-soluble extract from rosemary (*Rosmarinus officinalis*) containing rosmarinic acid, flavones and monoterpenes, enhanced hepatic metabolism and increased relative liver weight in rats (Debersac et al., 2001).

Digestive effects of herbs and spices, most of them indicated above, have been reported mainly in humans and laboratory animals, whereas few studies have been conducted on farm animals.

In the rabbit, the dietary inclusion of 0.5% fennel (*Foeniculum vulgare* Mill.) seeds with 0.5% oregano leaves from 5 to 13 weeks of age improved ($P < 0.05$) the organic matter, crude fibre and ether extract digestibility of diets that contained sunflower oil, whereas total cholesterol decreased (Omer et al., 2013). Gerencsér et al. (2014) reported that ether extract apparent digestibility was improved by the dietary supplementation of thyme (*Thymus vulgaris*), whereas starch digestibility improved with spirulina (*Arthrospira platensis*) plus thyme supplementation. As a drawback, spirulina plus thyme supplementation had a negative effect on cellulose and on crude protein digestibility, thus impairing the rabbit diet's digestible protein content. A recent study that added a herbal feed additive (Digestarom®) containing a mixture of onion, garlic, caraway, fennel, gentian, Melissa (*Melissa officinalis*), peppermint, anise, oak bark, and clove to the rabbit diet worsened the apparent digestibility of starch and cellulose (Celia et al., 2016). Diets supplemented with dry purple loosestrife (*Lythrum salicaria*) leaves (0.2% and 0.4%) or with 0.3% Cunirel® (a commercial herbal mixture containing purple loosestrife as the main ingredient) led to a decrease in nutrient digestibility when 0.4% loosestrife or 0.3% Cunirel® was added (Kovitvadhi et al., 2015). It therefore seems that herbal supplementation has no clearly positive effect on nutrient digestibility in the rabbit.

In early-weaned pigs, the incorporation of carvacrol, cinnamaldehyde and capsicum oleoresin promoted changes in digestive function and microbial ecology (Manzanilla et al., 2004), while herbal extract containing cinnamon, thyme, and oregano (*Origanum vulgare* L.) extract reduced the proliferation of coliform bacteria (Namkung et al., 2004). In addition, essential oil from oregano, cinnamon, pepper, sage (*Salvia officinalis* L.), thyme, and rosemary has been shown to improve apparent whole-tract and ileal digestibility in chickens (Hernández et al., 2004).

Table 1
Daily weight gain of growing rabbits fed diets enriched with herbs and spices.

Supplements	Daily weight gain			Authors
	Control g/day	Supplemented		
		g/day	Diff., %	
Digestarom®	45.7	54.5***	+19.3	Krieg et al., 2009
Ginseng extract, 0.03%	39.5	42.5*	+7.6	Chrastinová et al., 2009
L. albus, *T. foenum-graecum*, *C. senna*, 1.5%	30.4	31.6	+3.9	Omer et al., 2012
Ginseng extract, 0.03%	37.1	37.4	+0.8	Chrenková et al., 2013
Oregano leaves, 1%	32.0	34.5*	+7.8	Omer et al., 2013
Fennel seeds, 1%	32.0	35.7*	+11.6	Omer et al., 2013
Oregano leaves, 0.5%+ Fennel seeds, 0.5%	32.0	36.0*	+12.5	Omer et al., 2013
Sage leaves, 1%	23.9	23.6*	+11.2	Rotolo et al., 2013
Oregano, 1%	23.9	27.1*	+13.4	Rotolo et al., 2013
Spirulina, 5%	37.8	38.6	+2.1	Gerencsér et al., 2014
Thyme, 3%	37.8	39.1	+3.4	Gerencsér et al., 2014
Spirulina, 5%+ Thyme, 3%	37.8	38.1	+0.8	Gerencsér et al., 2014
Oregano extract, 0.2%	23.7	24.3**	+2.5	Cardinali et al., 2015
Rosemary extract, 0.2%	23.7	23.9	+0.8	Cardinali et al., 2015
Oregano extract, 0.1%+ Rosemary extract, 0.1%	23.7	25.0**	+5.5	Cardinali et al., 2015

Only effects marked with ***,**,* showed a significant improvement for $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

3.2. Growth promoter effect

Several studies on plant extracts as growth promoters have shown promising results in many animal species (see the review by Frankič et al. (2009)). As summarised in Table 1, in growing rabbits, when diets were supplemented with oregano leaves or oregano aqueous extract (at 1% or 0.2%, respectively), daily weight gain (DWG) increased significantly by 7.8–13.4% and 2.5%, respectively. Similar results were found using sage leaves (1%) or fennel seeds (1%), with improvement in DWG of 11.3% and 11.6%, respectively ($P < 0.05$) when compared to control diet. Rosemary extract (0.2%) was ineffective in promoting growth.

Positive effects on growth were also found when mixed compounds, such as oregano leaves (0.5%) and fennel seeds (0.5%) (+12.5% DWG, $P < 0.05$), or oregano extract (0.1%) and rosemary extract (0.1%) (+5.5% DWG, $P < 0.01$), were included in rabbit diets (Table 1). Also the mixture of *Lupinus albus*, *Trigonella foenum-graecum* and *Cassia senna* at 1.5% inclusion level was shown to improve the utilisation of a low energy diet (Omer et al., 2012).

Rabbit growth and feed conversion ratio were also enhanced through the dietary supplementation (300 mg/kg feed) of Siberian ginseng (*Eleutherococcus senticosus*) extract (Chrastinova et al., 2009) or 300 mg/kg feed of a commercial phytogenic feed additive

(Cuxarom Spicemaster) composed of a mixture of brown algae, basil, fennel, garlic, cinnamon, and essential oils from aniseed and thyme (Matusevicius et al., 2011). However, these two studies were not consistent with others that tested the same compounds (Chrenková et al. (2013) and Krieg and Rodehutscord (2004), respectively), and thus the effect of Siberian ginseng and Cuxarom Spicemaster on rabbit live performance is still uncertain.

One study reported positive effects of Digestarom® on productive performance (Abd-El-Hady, 2014). However this study is questionable because the sample sizes were very small, though the rabbits were group housed, individual feed consumption and feed conversion ratios were provided, and there was no indication of the analysed chemical composition of the experimental diets.

As with oregano, the different methods of use (in plant, botanical, extract, or essential oil), supplementation levels, and the mixtures employed do not permit clear identification of the effects these phytobiotics have on growing rabbit performances. For example, the efficacy of thyme in improving the live performances in rabbits has not yet been confirmed. Although thyme has been shown to increase palatability and feed intake in growing rabbits (Fekete and Lebas, 1983), recent studies testing dry thyme leaves and spirulina powder included separately or in combined form in diets fed at 2.5% and 3% to growing Dwarf rabbits (Dalle Zotte et al., 2013) or to hybrid rabbits at 3% or 5% (Gerencsér et al., 2014) did not demonstrate any substantial effect on growth performance or health status.

In poultry, mixtures of essential oils of cinnamon, oregano, thyme, cayenne pepper, citrus (Lippens et al., 2005) or oregano, laurel, sage, anise, citrus (Çabuk et al., 2006) improved feed conversion ratio. An essential oil combination derived from herbs growing wild in Turkey was found to have a beneficial effect on body weight, feed intake, feed conversion ratio, and carcass yield when used as a feed additive for broiler chickens (Alcicek et al., 2003, 2004). Also an experimental product containing 30% clove oil, at doses of 100–200 mg/kg feed, seems to improve feed efficiency in broiler chickens (Agostini et al., 2012). In piglets, garlic (Janz et al., 2007) or garlic plus cinnamon extracts (Zigger, 2001) proved capable of improving feed intake and daily weight gain. The dietary inclusion of essential oils or extracts from several herbs and spices in rabbits appears to have much less positive effects (Erdélyi et al., 2008) than those observed for broilers and piglets, likely due to the specific digestive physiology of the rabbit.

3.3. Galactagogue effect

Milk production is a complex physiologic process involving physical factors and the interaction of multiple hormones. Galactagogues are medications or other substances believed to assist with initiation, maintenance or increase of milk production (Bharti et al., 2012). In a global scale review, Bingal and Farnsworth (1991) documented over 400 plant species that have been used to facilitate lactation, the larger part of which were alleged galactagogues. Although most galactagogues have not been scientifically evaluated, traditional use suggests their safety and a certain degree of efficacy. In humans, anise, basil, fennel, mauve, verbena, cumin and grape have been traditionally used (Gabay, 2002).

To date, although many plant preparations, such as *Leptadenia reticulata, Asparagus racemosus, Withania somnifera, Arundo donax, Cissampelos pareira,* and *Foeniculum vulgare,* and extracts of *Eclipta alba* and *Solanum nigrum* have been incorporated in polyherbal formulations/tablets like Galog (Indian herbs), Ruchamax (Ayurvet), Payapro (Ayurvet), Leptaden (Alarsin vet), Calshakti Platina (Intas Pharmaceuticals), Ricalex (Aphali Pharmaceuticals), and Lactara (TTK Pharma) have been used around the world for their alleged galactogogues properties, the specificity and power of the galactopoietic effect of the individual plants still remain to be validated (Behera et al., 2013).

Among the natural products tested in farm animals, only a few have been shown to increase milk production including galega (Gonzalez-Andres et al., 2004) in sheep, fenugreek seed (Shah and Mir, 2004) and Silymarin, a standardized extract from seeds of milk thistle (*Silybum marianum* L.) in dairy cows (Tedesco et al., 2004), and fenugreek seeds (Alamer and Basiouni, 2005), galega and pea seeds (Spruzs and Selegovska, 2010) in goats. Recently, shatavari (*Asparagus racemosus*) has been shown to possess a lactogenic effect in dairy cows (Behera et al., 2013) in support of previous results with cows and buffaloes (Tanwar et al., 2008; Singh et al., 2012). Although the above-mentioned results indicate the galactagogue effect of some herbs or seeds, very little research has been conducted with rabbit does. Eiben et al. (2004) supplemented the diet with anise (6 g/kg) and fenugreek (6 g/kg) seeds, but the diet did not improve milk production or nursing performance in highly productive does. When Digestarom® was supplemented into the diet (300 mg/kg), in the initial period some rabbit does refused to consume the pellets and attempted to scrape them out of the feeders. Their performance declined as a result (Celia et al., 2015).

On the basis of these few studies and the divergent results obtained with farm animals, as suggested by Mortel and Mehta (2013) for women, well-designed, well-conducted studies are required to generate a sufficient body of evidence before recommending the use of herbal galactogogues with farm animals.

3.4. Antimicrobial and anticoccidial effect

Many herbs and spices have been recognized to possess antimicrobial and anticoccidial effects (Wilkins and Board, 1989) and traditional approaches for protecting livestock and food from disease, pests and spoilage in industrial countries are gaining momentum. The antimicrobial effect derives especially from plant essential (volatile) oils. Thymol and carvacrol, active components of many essential oils, disrupt cell membrane integrity, which further affects the pH homeostasis and equilibrium of inorganic ions (Helander et al., 1998; Lambert et al., 2001). For this reason, the volatile oils of black pepper, clove, geranium, nutmeg, oregano, and thyme (all of which contain carvacrol) are effective against *Enterococcus faecalis, Escherichia coli, Salmonella pullorum, Staphylococcus aureus,* and *Yersinia enterocolitica*, with the essential oil of thyme being the strongest inhibitor (Dorman and Deans, 2000). In addition, cinnamon volatile oils and their active compounds (cinnamaldehyde and eugenol) have shown antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Salmonella spp. and Vibrio parahaemolyticus* (Chang et al., 2001). Olive extract and its active compound oleuropein have also been proven to have antimicrobial effect against pathogens such as *Bacillus cereus, Staphylococcus aureus, Salmonella enteritidis,* and *Listeria monocytogenes* (Nychas et al., 1990; Tassou et al., 2000).

Antimicrobial effects have also been attributed to many other volatile oils (from lemon grass, laurel, black cumin seeds, anise, peppermint, onion, and garlic), mostly only through *in vitro* studies (see review of Krieg (2013)).

Even though digestive disturbances are responsible for substantial economic losses by rabbit farmers, research into the use of herbs and spices in an attempt to reduce rabbit morbidity and mortality is still scarce. The antimicrobial effects of some herbs are summarised in Table 2.

When Krieg et al. (2009) fed rabbit does and kits the commercial Digestarom®, a stabilizing effect on the microbiota was observed that resulted in a significantly lower prevalence and severity of digestive disorders after weaning (Table 2). A similar

Table 2
Claimed antimicrobial effects of dietary supplementation with herbs and spices on growing rabbits.

Supplements	Results	Authors
Digestarom®	Stabilizing effect on microbiota	Krieg et al., 2009
Black cumin seeds (2 or 9%)	Influence on resistance against Pasteurellosis	El-Bagir et al., 2010
Spirulina (5%)+ Thyme (3%)	Decreased the number of *C. coccoides* and *C. leptum*	Vántus et al., 2012
Thyme essential oil, 0.5 g/kg	Intestinal barrier strengthened and limited the growth and colonisation of pathogenic bacteria	Plachá et al., 2013

effect was observed by dietary supplementation with 0.5 g/kg DM thyme essential oil, which improved intestinal integrity and displayed a tendency to stimulate the abundance of certain beneficial microbes in the rabbit gut (Placha et al., 2013; Table 2).

Although essential oil of thyme showed antimicrobial action both *in vitro* (Dorman and Deans, 2000) and *in vivo* (Placha et al., 2013), another *in vivo* study on growing rabbits fed a supplement of 3% thyme leaves did not exhibit a substantial effect on volatile fatty acid (VFA) production or caecal microbiota composition (Vántus et al., 2012; Table 2). Surprisingly, when diets were supplemented with 3% thyme leaves and 5% spirulina, an antimicrobial effect on the bacterial groups investigated in the caecum (*C. coccoides, C. leptum*) was noticed (Vántus et al., 2012; Table 2), although the authors suggested testing the effects of spirulina and/or thyme on health status under poorer sanitary conditions.

Black cumin (*Nigella sativa*) seed oil fraction contains thymoquinone, which proved to exert anti-inflammatory, anti-bacterial and immunomodulatory effects *in vitro*. When it was fed at either 2 or 9% in the diet of laboratory rabbits aged 3–4 months, it was shown to stimulate their immune system and to extend their survival time after intraperitoneal administration of *Pasteurella multocida* (El Bagir et al., 2010, Table 2).

In piglets, active substances from garlic have been shown to reduce the incidence of infection from *E. coli* and to suppress the action of fungi and viruses (Zigger, 2001). In weaned pigs, the antibacterial activity of cinnamaldehyde (one of the active compounds of cinnamon) has been shown to be effective in improving health and live performances (Zigger, 2001). Garlic or cinnamon extract or their active compounds have not yet been tested as antimicrobials on growing rabbits.

In the rabbit, the search for natural alternatives to anticoccidials is very important. However the research is still scarce, however, and the studies conducted using herbs and spices supplemented as plants or botanicals have not provided promising results. Some positive results have recently been observed when supplementing diets with extracts of oregano and garlic oils at concentrations ranging between 0.5–1 g/kg diet (Kowalska et al., 2012).

Based on *in vivo* studies on other species, some herbs have been reported to be effective in treating coccidiosis in poultry, such as *Dichroa febrifuga* and *Sophora flavescens*, both of which are important in traditional Chinese medicine (see the review by Frankič et al. (2009)). Recently, three other phytogenic products were tested (oregano; a mixture of Curcuma, saponins and inulin; and *Quillaja saponaria*) to alleviate negative effects in coccidiosis-challenged broiler chickens, but none were shown effective (Scheurer et al., 2013).

3.5. Antioxidant effect of herbs and spices

The demand for antioxidants of plant origin capable of replacing synthetic antioxidants in feeds and foods has increased considerably in recent years, and for this reason the health and antioxidant properties of many herbs and spices are currently undergoing scientific investigation (Kaefer and Milner, 2008; Shah et al., 2014).

Many herbs and spices contain active components capable of exerting antioxidative action, such as phenolic substances (flavonoids, tannins, phenolic acids, and phenolic diterpenes) and vitamins E, C and A. These plants or their extracts can be used for a triple purpose in animal feeding and food technology: protecting animal feed against oxidative deterioration during storage, protecting tissue integrity in live animals, and enhancing the oxidative stability of meat and meat products during storage or ripening. For the latter purpose, herbs and spices (oregano, rosemary, sage, thyme, cinnamon, tea, mint, ginger, clove, *etc.*) or their extracts (prepared from the plant material) can be also directly added to the meat products during processing.

Dalle Zotte and Szendrő (2011) reviewed the antioxidant effect of the herbs and spices that had been tested. Additional research has recently been conducted to evaluate the effect on oxidative stability of rabbit meat provided by the dietary supplementation of herbs and spices (Table 3).

Oregano essential oil contains monoterpenes, thymol, and carvacol, all of which have antioxidant and antimicrobial properties that have been proven both *in vitro* and *in vivo* (Vekiari et al., 1993; Kulisic et al., 2004; Shan et al., 2005; Coma, 2008; Zinoviadou et al., 2009).

Supplementing the rabbit diet with oregano essential oil (200 mg/kg; Botsoglou et al., 2004) or extract (2 g/kg; Cardinali et al., 2015) has been shown to improve the oxidative stability of rabbit meat, providing indirect evidence that antioxidant compounds in oregano essential oil are absorbed by the rabbit gut, thus increasing tissue antioxidant capacity. Furthermore, dietary enrichment with oregano essential oil (200 mg/kg) increased rabbit meat shelf-life by reducing average microbial counts on carcasses in 12-d refrigerated storage (Soultos et al., 2009).

Rosemary also contains a high level of phenolic antioxidants. Rosemary extract, supplemented alone (2 g/kg) or combined with oregano extract (1 g/kg each), was also shown to be effective in delaying lipid oxidation in rabbit loin meat, but less efficaciously than oregano (Cardinali et al., 2015; Table 3). On the other hand, oregano or sage dry leaves at 1% dietary inclusion level (Rotolo et al., 2013) did not provide successful antioxidant protection in rabbit loin meat. The authors indicated the herb extraction technique and dietary dose as the main causes.

Dietary supplementation with chia seed (*Salvia hispanica*) dietary supplementation (15%) was also ineffective in preventing lipid oxidation in ground rabbit hindleg meat due to the increased PUFA level produced by chia supplementation (Meineri et al., 2010).

Thyme contains thymol and carvacrol, which are considered to possess strong antioxidant activity. Some recent evidence does not entirely support this effect in rabbits, however. The oxidative stability of raw rabbit loins supplemented with 3% thyme leaves was confirmed during nine days of retail display (Dal Bosco et al., 2014; Table 3). This effect was not seen in raw or cooked hindleg meat (Dalle Zotte et al., 2014a; Table 3). Further studies would be required to confirm the antioxidant activity of the bioactive compounds of thyme leaves by increasing the dietary inclusion level of this herb as raw material.

Green tea (*Camellia sinensis*) is a traditional, popular beverage that promotes health by preventing lipid oxidation due to the effect of the predominant group of polyphenols (catechins) contained in its leaves (Zhonga et al., 2009). Eid et al. (2010) reported that feeding rabbits diets containing 0.5% of green tea significantly decreased thiobarbituric acid-reactive substances (TBARS) in rabbit hind leg and loin meat stored for two months but did not affect

Table 3
Oxidative stability (TBARS values, mg MDA/kg meat) of meat from rabbits fed diets enriched with herbs and spices.

Supplements	Control	Oregano leaves	Oregano extract	Sage leaves	Rosemary extract	Oregano extract + Rosemary extract	Reishi mushroom	Spirulina	Thyme	Spirulina + Thyme	Authors
Inclusion level		1%	0.2%	1%	0.2%	0.1%+0.1%	1%	5%	3%	5%+3%	
Raw-Fresh	0.30	0.45		0.43							Rotolo et al., 2013
Raw-Fresh	0.24		0.18**		0.21**	0.20**					Cardinali et al., 2015
Raw-Fresh	0.65							0.63	0.54	0.79	Dalle Zotte et al., 2014a
Cooked-Fresh	1.73							1.99	1.25	1.80	Dalle Zotte et al., 2014a
Raw 1 d	0.15							0.18	0.14	0.16	Dal Bosco et al., 2014
Raw 3 d	0.23							0.22	0.15*	0.24	Dal Bosco et al., 2014
Raw 6 d	0.26							0.24	0.17*	0.27	Dal Bosco et al., 2014
Raw 9 d	0.30							0.29	0.20***	0.27	Dal Bosco et al., 2014
Raw-Fresh	0.27						0.19**				Trebušak et al., 2014
Cooked-Fresh	0.83						0.69***				Trebušak et al., 2014
Raw 6 d	1.01						0.60**				Trebušak et al., 2014
Cooked 6 d	0.87						0.72***				Trebušak et al., 2014

* The differences between control and treated groups were significant at $P < 0.05$.
** The differences between control and treated groups were significant at $P < 0.01$.
*** The differences between control and treated groups were significant at $P < 0.001$.

the total reactive antioxidant potential values of rabbit serum. These results would confirm the hypothesis of different mechanisms of action exerted by the different antioxidants in various vegetal essences (scavenger *in vivo*, chain-breaking in membrane, *etc.*).

Trebušak et al. (2014) examined the effect of the dietary supplementation of 1% *Ganoderma lucidum* (Reishi mushroom) on the oxidative stability of rabbit meat. The MDA (malondialdehyde) values in the meat processed in different ways (fresh, stored, raw, cooked) were slightly lower in the treated groups than in the control group, but these differences were not statistically significant (Table 3).

It has also been suggested that the high number of potential antioxidants contained in plants probably act synergistically (McCarthy et al., 2001; Collin, 2006). This was demonstrated by Al-Jowari (2012), who revealed that providing laboratory rabbits with a diet containing 20% of a mixture of cinnamon, turmeric and cumin powder (*Cuminum cyminum*) in a 1:1:1 mixture ratio provided beneficial effects at oxidative stress conditions and reduced the risk of diabetes and atherosclerosis diseases by improving glucose and lipid metabolism. Excellent protection against oxidative stress was recently shown by *Coriandrum sativum* extract administered alone to laboratory rabbits, however (Joshi et al., 2012).

The results obtained seem to indicate the promising effects of diets enriched with selected herbs and spices in preventing or delaying lipid oxidation. Further research is required to study the effects of different levels or combinations of these natural antioxidants on the oxidative stability of rabbit meat.

4. Herbs and spices in meat and meat products

Herbs and spices have been proven to be effective in preserving and improving the quality of meat and meat products, acting mainly as antioxidants. Oxidative processes are one of the primary mechanisms of quality deterioration in meat and meat products because they worsen flavour, colour, and nutritive value, and consequently limit shelf-life (Kanner, 1994).

Spices such as clove and cinnamon, and herbs such as oregano, rosemary, and sage have been reported as playing major roles (Shan et al., 2005; Wojdylo et al., 2007; Karre et al., 2013), while also reducing colour loss and microbial growth (Djenane et al., 2002, 2003; Coma, 2008; Zinoviadou et al., 2009) in some types of red meat. Furthermore, the use of melissa was found effective in preventing lipid oxidation in new formulations of reduced-fat Bologna type sausage (Berasategi et al., 2014). Recently, Lorenzo et al. (2014) reported that green tea extract seems to offer a promising alternative to commercial antioxidants for extending the shelf-life of pork patties to 20 d of refrigerated storage.

As previously mentioned, garlic is one of the most common cooking ingredients, and allicin, which is one of its compounds with antimicrobial properties, has been shown to be effective, both in fresh and powder form, in chicken sausages, thanks to its combined antimicrobial and antioxidant effects (Sallam et al., 2004).

One emerging natural source of unique phenolic compounds, such as aspalathin, is a South African leguminous shrub named rooibos (*Aspalathus linearis*). Cullere et al. (2013) tested three forms of unfermented (green) rooibos: dried leaves, water extract, and freeze-dried extract when added at 2% inclusion level to ostrich meat patties on an 8-d shelf-life trial. The authors observed that rooibos considerably lowered ostrich patty TBARS content, in this way extending shelf-life. The same authors (Cullere et al., 2013) also tested the addition of different concentrations (0%, 0.25%, 0.5% and 1%) of a fermented rooibos extract to nitrite-free

ostrich salami. The higher inclusion levels (0.5% and 1%) were also effective in delaying lipid oxidation in ostrich salami until 15 d of ripening.

Considering the high amount of PUFA in rabbit meat and the increasing popularity of rabbit meat sold in ready-to-cook and serve retail packs, researchers have begun to focus on the problem of preventing (or limiting) lipid and protein oxidation during storage (Dalle Zotte and Szendrő, 2011).

To date, only a few studies have tested the use of herbal products in prolonging rabbit meat shelf-life. Fermented rooibos tea extract was included in rabbit meat patties at 0.5%, 1% , and 2% concentrations in a 6 d shelf-life trial. At all inclusion levels, fermented rooibos was found capable of lowering peroxide content compared to all untreated meat samples (Dalle Zotte et al., 2014b), thus confirming the antioxidant potential of fermented rooibos in rabbit meat. In addition, fermented rooibos tea extract at 0.5% inclusion level guaranteed the same general product acceptability as the control rabbit meat patties, thus suggesting the potential use of this plant as a natural additive in the meat sector (Cullere et al., 2015). To improve the microbiological quality and extend the shelf-life of refrigerated rabbit meat, Ali et al. (2015) treated the meat with lactic acid (0.5%), thyme oil (0.5%) and water extract of sumac (*Rhus coriaria* L.) (8%) by dipping for 1, 1 and 10 min in each of the treatments, respectively. Sumic and lactic acid extended the shelf-life of rabbit meat for about 3 and 6 d in comparison with control and thyme oil groups during refrigerated storage at 2 °C, respectively.

5. Conclusions

Following the European ban on the use of antibiotic growth promoters in animal feeding, researchers have increased the intensity of the search for natural additives suitable for use as probiotics and prebiotics, and enzymes, organic acids, herbs, spices, and botanicals capable of improving farm animal health status and production. Several herb, spice, and botanical products have been tested on different animal species. When supplemented to diets, some have shown beneficial effects in rabbit production as growth promoters (oregano seeds or leaves, sage leaves, fennel seeds), antimicrobials (thyme essential oil or leaves, black cumin), and antioxidants (oregano essential oil or extract, thyme leaves, green tea leaves). Rabbit meat shelf-life has been extended by supplementing fermented rooibos tea extract to fresh meat, whereas other herbs, spices or botanicals have not yet been tested. Research on the use of herbs or/and spices has demonstrated their potential as feed additives and/or antioxidants, but further research is recommended to optimize effects on rabbits before practical proposals can be drafted.

Conflict of interest

There is no conflict of interest among the authors.

Implications

Increased consumer awareness and consumption of safe, natural foods prompted research for alternative animal feeding strategies able to replace antibiotic growth promoters (AGPs) and synthetic antioxidants. Studies recently conducted on rabbits have reported benefits to animal health and performance, and to the nutritional value and shelf life of the meat. This review summarises the results obtained in the dietary use of herbs and spices in rabbits and their inclusion in fresh or processed meat.

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2.1 Description and functions of *Silybum marianum* and of a commercial product (Digestarom®) derived of a mix of selected herbs

Silybum marianum is an herbaceous plant of Asteraceae family, that commonly grows in the Mediterranean countries. The plant is popularly famous as milk thistle because a legend tells how the plant obtained its aspect from a drop of Virgin Mary, while she was nursing Infant Jesus. The major active compound of *S. marianum* is the silymarin, a standardized mixture of seven flavonolignans that represent 65-80% of the plant: silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristrin, and silydianin, and one flavonoid (taxifolin) (Colturato et al., 2012). The fruit and the seeds possess higher percent of the active compounds, despite is present in the full plant too (Flora et al., 1998; Šeršeň et al., 2006; Engelberth et al., 2008). In humans, the *S. marianum* is considered an important medicinal crop, and, in Europe,it is mainly prescribed to treat the disorders (Rambaldi et al., 2005) and chronic disease of the liver (Freedman et al., 2011). However, *S. marianum* is supposed to have choleric and anti-inflammatory (Guptya et al., 2000) properties, functioning as lipid peroxidation inhibitor (Nencini et al., 2007; Veknin et al., 2008), promoting liver cell regeneration, and reducing blood cholesterol content (Giese et al., 2001). In addition, it exhibited antioxidant properties both *in vitro* and in a rat animal model (Šeršeň et al., 2006; Nencini et al., 2007).

Studies on the dietary inclusion of *S. marianum* to broiler chickens , showed its benefit on productive performances, immune system, carcass characteristics and meat quality (Kalantar et al., 2014, Kralik et al., 2014; Morovat et al., 2015, Zarei et al., 2016).

In the growing rabbit, a recent study demonstrated that dietary inclusion of *S. marianum* fruits (1%) was able to attenuate their mortality (Kosina et al., 2017).

Digestarom® 1315 is a commercial herbal formulation designed as a rabbit feed supplement made of a mixture of ten different ingredients (Colin *et al*., 2008): onion (*Allium cepa L.)*, garlic (*Allium sativum L.*), caraway (*Carum carvi L.*), fennel (*Foenicum vulgarae L.*), gentian (*Gentiana lutea L.*) melissa (*Melissa officinalis L.*), mint (*Mentha arvensis L.*), anise (*Pimpinella anisum L.*) oak bark (*Quercus cortex*) and clove (*Syzygium aromaticum L.*). Such herbal formulation was previously tested by some authors on growing rabbits whose found positive effects, such as reduction in mortality and improvement of the final body weight, attributed to the high content of phenols and flavonoids substances in the ingredients (Colin et al., 2008; Krieg et al., 2009; Abd-El-Hady et al., 2013; Abd-El-Hady, 2014). Studies on the single plants have also reported several positive effects. Dietary supplementation of onion (Gugolek et al., 2008) and mint (Mahmoud, 2015) improved productive performances of rabbits, whereas broiler chicks increased the body weight when fed with garlic fermented by-products (Kang et al., 2010) or when 1, 2 or 3 g/kg of fennel seeds were added to the diet (Abdullah and Abbas, 2009). In broilers dietary supplementation of garlic improved the carcass and breast yield with enhancement of meat texture and flavour (Raeesi et al., 2014).

Anise and fennel essential oil improved the body weight of turkey when added to the diet (Yacoub et al., 2015) whereas fennel essential oil supplemented alone reduced the mortality in growing rabbit (Benlemlih et al., 2014). Spices known for their stimulant effect on appetite are clove,

caraway and gentian (Baytop, 1984; Loo and Richard, 1992). Due to its bitterness, gentian root increased saliva and digestive juices secretions thus alleviating digestive disorders in dogs (Meir and Meier-Liebi, 1993). Clove essential oil improved the final body weight and breast yield of chicken broilers (Isabel and Santos, 2009). As for *Melissa officinalis*, it was able to significantly reduce lipid oxidation in chicken breast and thigh (Kasapiou et al., 2014), whereas its essential oil lowered the lipid level in rabbit fed with cholesterol-increased diet (Karimi et al., 2010). Oak bark is traditionally used in humane consumption to treat digestive problems but the high content of tannins provoke astringency (Łukasz Łucza et al., 2014; Gonultas and Ucar, 2017).

Table 1 summarises all the results obtained by the single use of the above mentioned herbs and spices. Since many positive effects were observed, it was supposed that their combination in a unique dietary supplement to growing rabbits would have enhanced their benefits. Thus, the purpose of the study conducted in this PhD thesis was to evaluate the effect of the dietary inclusion of Digestarom® on growing rabbits health, nutrients digestibility, caecal and faecal microbial population count, live performances, carcass and meat quality.

Tabella 1 Effect of single herbs and spices included in Digestarom® and Silybum marianum

		Effect				
Herbs and spices	Milk thistle	X	X		X	X
	Melissa					X
	Gentian		X	X		
	Fennel	X	X			
	Mint	X				
	Clove	X	X	X		
	Oak bark					
	Anise	X				
	Caraway			X	X	
	Garlic	X	X		X	
	Onion	X				
		Increase body weight	Improve carcass yield	Appetite stimulant	Improve meat quality	Antioxidant activity
		chicken, turkey, rabbit	chicken	Turkey, dog	Chicken, rabbit	Rat, chicken, rabbit

3. AIMS OF THE PhD RESEARCH

The European antibiotics ban in 2006 forced research institutions and private companies to find suitable alternatives to control animal health.For this reason, several studies to find the best alternative solution were conducted recently. Herbs and spices are considered a good alternative since they were used for millennia in human remedies and nutrition, and are generally recognised to have an healthy effect due to the presence of the so-called phytochemicals.

The aim of this PhD thesis was to study the effect of the dietary supplementation of a single herb, or of a mix of selected herbs and spices on the productive performances, health status and meat quality of growing rabbits.

Indeed, the first study aimed to investigate in depth the effects of dietary supplementation of Digestarom® (a commercial product made of a mix of 10 herbs and spices) on the total tract apparent digestibility, faecal and caecal microbial counts, live performance and health status of growing rabbits measured at different times during the growing period. For the first time, the effects of before and after weaning supplementation on the live performance of growing rabbits were considered.

The second study evaluated the effect of Digestarom® on carcass traits and rheological and sensory meat quality.

The aim of the third study was to study the effect of a dietary supplementation of a dried powder of *Silybum marianum* on the live performances of growing rabbits, their health status and carcass traits. In addition, quality and sensory properties of the derived meat were evaluated.

4. METHODOLOGY SUMMARY OF THE DISSERTATION

With this PhD thesis the effect of dietary inclusion of a mix of plant (Digestarom®) or a single plant (*Silybum marianum*) to growing rabbits on their health status, live performances, carcass and meat quality, and on nutrients digestibility and gut health was investigated. All studies did not consider antibiotics supplementation.

The following section reports material and methods used in the three experiments. The first 2 sub-chapters summarises information about the animals used, the experimental design, data collection and management of the three experiments, whereas the other methodologies, peculiar for single experiment, are reported in 3 different sub-chapters.

4.1 Animals and experimental design

Experiment 1 was carried out in the experimental farm of Kaposvár University. The animals derived from a previous part of the experiment which also aimed to evaluate the effect of dietary supplementation with Digestarom®, a commercial product, on the reproductive performance of rabbit does (Celia et al., 2015). At kindling, does and litters were divided in 2 dietary groups and fed with balanced pelleted diets without antibiotics: the first group (51 does/group) received a commercial diet (group C), whereas the other one (52 does/group) was fed the same diet supplemented with 300 mg/kg of Digestarom® (group D). However, the litters were fed experimental diets from 21st d of life onward. This represented the Before Weaning phase (BW), described in a previous article (Celia et al., 2015). At weaning (35 d), each group was further

divided into 3 feeding groups: CC rabbits received the C diet and DD ones received the D diet from 5 to 12 wk of age. Differently, DC rabbits were fed with D and C diets from 5 to 8 wk of age and from 8 to 12 wk of age, respectively (Figure 1). This represented the After Weaning phase (AW). The experiment involved 372 growing rabbits of the Pannon breeding programme (Pannon Ka maternal line). Among them, 324 rabbits were used to evaluate the growth performance (54 rabbits/diet), whereas 48 rabbits were used for gastrointestinal pH and caecal microbial count analyses. From the 48 rabbits, 12 were slaughtered at 5 wk of age (6 rabbits/diet) and 36 were reared separately, then 24 were slaughtered at 8 wk of age (6 rabbits/diet). Remaining rabbit were not considered for the study. The kits were housed in wire-mesh cages (3 rabbits/cage, size of cage: 61x32x30 cm). The temperature and the photoperiod were 15-18°C and 16 h light: 8 h dark, respectively.

In experiment 2, animals from experiment 1 were used for carcass measurements and meat quality analysis. .

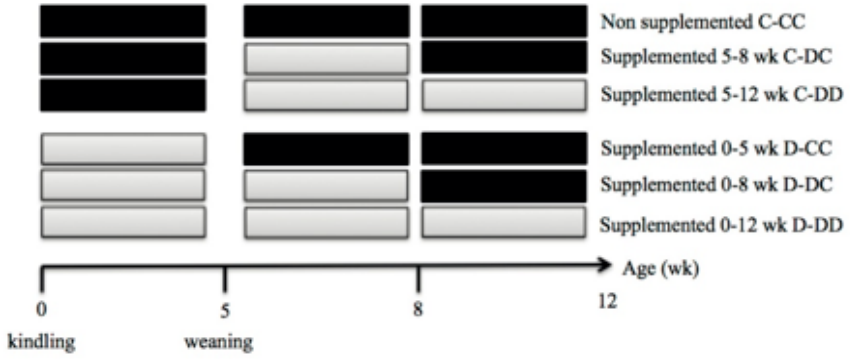

Figure 1: Experimental design (n=54 rabbits/treatment). ▢ Diet D, supplemented with 300 mg Digestarom®. ■ Diet C.

In experiment 3, a total of 144 Pannon Large rabbits (both sexes) of the Pannon Breeding Program were involved in the experiment in the experimental farm of Kaposvár University. At weaning (35 days of age),

animals were divided into three feeding groups: the control group (C, n=51 rabbits) was fed a basal diet, whereas the other two groups received the control diet supplemented with two different concentrations of dried *Silybum marianum* (SM) which were 5 g/kg (SM1, n=48) and 10 g/kg (SM2, n=45). All diets had no anticoccidials or any other medications. The product was obtained from Johannesburg University and previously used in Marie Curie project named "herbal protection". Morbidity (diarrhoea, unkempt fur, bloody faeces and respiratory problems) and mortality were recorded daily. Animals were housed in wire-mesh cages (3 rabbits/cage, size of cage: 61x32x30 cm). The temperature and the photoperiod were 15-18°C and 16 h light: 8 h dark, respectively.

4.2 Performance data collection and management

In experiment 1 body weight of rabbits was measured at 5, 8 and 12 wk of age, feed intake for 5-8 and 8-12 wk periods was recorded and the daily weight gain and feed conversion ratio were then calculated. Body weight and daily weight gain were evaluated based on individual data, whereas feed intake and feed conversion ratio were based on the cage unit. When calculating feed intake, it was assumed that morbid rabbits did not consume any pellets for the 2 d preceding their death. Mortality was recorded daily.

In experiment 2, at 12 weeks of age, rabbits from the experiment 1 were transported to a slaughterhouse located 200 km from the experimental farm. After fasting (6 h, inclusive of 4 h for transportation) and electro-stunning, rabbits were slaughtered by cutting the carotid arteries and jugular veins. Carcasses were dissected according to World Rabbit Science Association (WRSA) recommendations as described by Blasco &

Ouhayoun (1996). The slaughtered rabbits were bled, and then the skin, genitals, urinary bladder, gastrointestinal tract, and the distal part of legs were removed. Warm carcasses (with head, set of organs consisting of the thymus, trachea, oesophagus, lung, and heart, liver, kidneys, and perirenal fat and scapular fat) were weighed and the ratio to slaughter weight (SW) was calculated. Carcasses were then chilled at +4 °C for 24 h. The chilled carcasses (CC) were then weighed. The head, set of organs, liver, and kidneys were removed from each carcass to obtain the reference carcass (RC),which included the meat, bones, and fat deposits. The carcasses were then cut between the 7th and 8th thoracic vertebra and between the 6th and 7th lumbar vertebra to obtain the fore, mid, and hind parts, which were weighed separately. The ratio of the head, organs, fat deposits, and carcass parts to either CC or RC weights were calculated as required. Hind legs (HL, right and left) and Longissimus thoracis et lumborum (LTL) muscles were dissected from 15 rabbits per dietary treatment (N = 90 rabbits) and weighed. They were then individually packed in polyethylene bags (water vapour transmission rate: 3.5 ± 1 g/m2·day at 23 °C and 85±2% R.H.), vacuum-sealed using a CSV-41n ORVED machine (99% vacuum level), and ice-cooled in portable refrigerators. The next day, samples were transported to the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy) for meat quality analyses. During transport, the temperature of the sampleswas kept at 4±1 °C. The samples arrived at the MAPS Department around 33 h post-mortem and stored in a professional ventilated refrigerator at 4 ± 1 °C. The only exceptions were the right LTL and right HL, which were immediately stored at −40 °C until further analyses.

In experiment 3, animals were fed the experimental diets *ad libitum* from 5 to 11 wk of age.Body weights (BW) and average weight gain (AWG) were recorded based on the individual rabbit, whereas feed intake (FI) and feed conversion ratio (FCR) were calculated on the cage basis. Morbidity (diarrhoea, unkempt fur, bloody faeces and respiratory problems) and mortality were recorded daily. When calculating feed intake, it was assumed that morbid rabbits did not consume pellet for the two days before their death, hence they were not included in the feed intake calculations. At 11 weeks of age the animals undergo the same slaughter and carcass dissection described for experiment 2.

4.3 Experiment 1: Digestarom® productive performances.

4.3.1 pH of the stomach and caecal content and caecal microbial count

From 13:00 to 14:00 h six healthy rabbits per experimental group were slaughtered at 5 (6 C and 6 D) and at 8 wk of age (6 C-C, 6 C-D, 6 D-C, 6 D-D). The digestive tract of each animal was removed immediately and the stomach, small intestine and caecum were separated. The pH values of the stomach and caecal contents were determined using an OP-110, Radelkis pH-meter (Hungary).

From the 1 g sample taken from the caecal digesta of each rabbit (serial dilutions were made: 1 g caecal sample+9 mL diluent [0.9% NaCl]), and used for microbiological determination. Anaerobic conditions were ensured by the use of carbon dioxide.

The obligate anaerobe microorganisms were cultured on Schaedler's agar (Sharlan Chemie, Barcelona, Spain), the selectivity of which was

increased by the addition of esculin (Merck, Darmstadt, Germany), neomycin (Merck, Darmstadt, Germany) and iron ammonium citrate (Sharlan Chemie, Barcelona, Spain). Gamma sterile Petri dishes (Biolab, Budapest) were placed into Anaerocult culture system (Merck, Darmstadt, Germany), in which the anaerobic conditions were ensured by an "Anaerocult A" (Merck, Darmstadt, Germany) gas-producing bag. Subsequently, the samples were incubated in an LP 104 type thermostat (LMIM, Esztergom, Hungary) at 37°C for 96 h.

Total aerobic bacteria were cultured on media supplemented with 5% calf blood. The samples were incubated at 37°C for 72 h. E. coli and other coliform bacteria were cultured on a Chromocult differentiation medium (Merck, Darmstadt, Germany). The samples were incubated at 37°C, under aerobic conditions, for 24 h.

After the incubation time had elapsed, the colonies were counted according to standard methodology (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem). The colony counts were expressed in log10 colony-forming units (CFU) related to 1 g of sample.

4.3.2 Digestibility trial

An in vivo digestibility trial was carried out at the MAPS Department (Italy) according to the European standardised method (Perez et al., 1995). To this end, twenty 50 d-old growing rabbits were used to determine the total tract apparent digestibility (TTAD) of C and D diets (10 rabbits/diet). These rabbits received the C or D diets during the digestibility trial, only. Animals were equally distributed by gender and live weight (average live weight of 1478±142 g) into the 2 dietary groups and individually caged. After 1 wk of adaptation to the new diets, faeces

were collected for a 4-d period. Morbid and/or dead rabbits were excluded from the trial; they were not replaced and not considered in the statistical analysis.

The TTAD of dry matter (DM), organic matter, crude protein, ether extract, starch, neutral detergent fibre, acid detergent fibre, cellulose, hemicelluloses and gross energy of the experimental diets (C and D) was measured.

The day after the end of the digestibility trial, the rabbits continued to be fed the same experimental diets. Samples of hard faeces were collected from each animal and immediately submitted to the quantitative determination of coliforms, lactic acid bacteria and spore-forming aerobes (*Bacillus* spp.). Coliforms were counted using the same procedure previously reported for caecal content. The lactic acid bacteria load was measured by plating on MRS agar (Scharlan Chemie, Barcelona, Spain) after anaerobic incubation at 37°C for 48 h. The count of spore-forming Bacillus spp. was determined by plating on *Bacillus* Selective Agar (Oxoid LTD, Basingstoke, Hampshire, England) after aerobic incubation at 37 °C for 24 h. Colony counts were expressed in log10 CFU related to 1 g of sample.

4.3.3 Chemical analyses

Chemical composition of the experimental diets and faeces was analysed at the laboratory of the MAPS Department (Italy) in duplicate by AOAC (2000) methods to determine the concentrations of dry matter (Method no. 934.01), crude protein (Method no. 2001.11), crude fibre (Method no. 978.10) and ash (Method no. 996.11). Ether extract was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF without sodium

sulphite), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to Mertens (2002), AOAC (2000, Method no. 973.187), and Van Soest et al. (1991), respectively, using the sequential procedure and filter bag system (Ankom Technology, New York). The gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO, 1998). The mineral profile (Ca, P, K, Mg, Na, S, Fe, Zn) of the experimental diets was analysed by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10).

4.3.4 Statistical analysis

Digestibility data, faecal microbial count during the digestibility trial and caecal microbial count of rabbits at 5 wk of age were analysed by one-way ANOVA of the GLM procedure of the Statistical Analysis System (SAS Institute, 2004). Experimental diets (C, D) were considered as fixed effect. Live performance and caecal microbial count of rabbits at 8 wk of age data were subjected to another ANOVA in which a PROC MIXED procedure tested the effect of dietary supplementation before weaning (BW), after weaning (AW) and their interaction (BW x AW) on the studied variables. Microbial count data were also analysed by one-way ANOVA with age (5 and 8 wk of age) as fixed effect. Mortality data were analysed by chi-square test according to the Marascuilo (1966) procedure. Post hoc pairwise contrasts were evaluated by Bonferroni adjustments.

4.4 Experiment 2: Digestarom® meat quality

4.4.1 HL and LTL pH, colour, thawing and cooking losses, shear force values, and bone traits

Raw left *Longissimus thoracis et lumborum* muscle (LTL) and *Biceps femoris* muscle (BF) of the hind leg (HL) were used to measure the pH 48 h *post-mortem* using a Mettler Toledo FE20 pH-meter. Colour values of lightness (L*), redness (a*), yellowness (b*), chroma (C*) and hue H° (CIE, 1976) were subsequently measured on the same portions using a RM200QC colorimeter (X-Rite, Co, Neu-Isenburg, Germany. Measuring Area: 8 mm; Measuring Geometrics: 45/0 Image Capture; Illuminant/Observer: D65/10). The values adopted are the average of two measurements for each sample. Raw left LTL and HL were then individually packed in polyethylene bags, vacuum-sealed, and stored at −40 °C.

Right LTL and HL meat samples were allowed to thaw overnight at +4 °C, removed from plastic bags, weighed, and subsequently used for thawing and cooking loss determinations. For this purpose, LTL and HL samples were individually vacuum-packed in PVC bags and cooked in a water bath at 80 °C for 1 h and at 85 °C for 2.5 h, respectively. Warner-Bratzler Shear Force (WBSF) was assessed with a London,UK) on six cylinder-shaped cooked right HL meat pieces per sample (Ø 1.25 cm) sliced perpendicularly to the fibre direction by a Warner- Bratzler cell (100-kg load cell, 2 mm/s crosshead speed) inserted in the texturometer. The WBSF values of each sample are an average of the 6 measurements.

Left HL were thawed under the same procedure used for right HL, and deboned in order to determine the meat/bone ratio (Blasco & Ouhayoun, 1996). Femur and tibia were separately weighed, and then length and minor diameter were measured with a digital calliper (JUWEL Digital-

Schieblehre Rostfrei H4215/5X A12) before their incidences on HL were calculated. Femur fracture toughness (FT) was calculated at the average bone length point, corresponding to the mid diaphysis, using a dynamometer Texture TA-HD (SMS- Stable Micro System) with a 6 cm wide cell and a load rate of 0.5 mm/s.

4.4.2 Sensory analysis

After 2 months of frozen storage at −40 °C, the 90 left LTL (15 LTL per treatment) were subjected to a ranking test conducted by a trained four-member MAPS Department panel.

In order to familiarize with the food matrix and to select the appropriate descriptors, panel members underwent four pre-test training sessions, testing one rabbit loin/panellist/training session, purchased in a local supermarket. During the last two training sessions, panellists were also trained to familiarize with the ranking test and with the perception of the herbs and spices constituting Digestarom®, which were bought in a herbalist shop.

The test was carried out in 3 consecutive days in which 30 samples/ day were evaluated (5 samples × 6 treatments). Samples were identified by a random three-digit code, vacuum-sealed by 6 in PVC bags (DCC, D-DC, D-DD, C-CC, C-DC, C-DD) and cooked in a water bath at 84 °C until core temperature reached 74 °C every day of sensory analysis after thawing for 16 h at+4 °C. Each cooked meat sample was cut into four numbered pieces of equal size designated to a specific panellist and served still warm for the evaluation of sensory attributes. For each descriptor (olfactory rancidity, olfactory spicy, flavour rancidity, flavour spicy, overall acceptability), meat samples were ranked from least (rank

1) to most intense (rank 6). Lastly, the panellists were also asked which of the ingredients in Digestarom® (onion, garlic, caraway, TA-HDi Texture Analyzer (Stable Micro System, fennel, gentian, melissa,mint, anise, clove and oak bark) they could recognize (if any).

4.4.3 Statistical analysis

Data were analysed using SAS 9.1.3 statistical analysis software for Windows (SAS, 2008). Carcass and meat quality were subjected to an ANOVA MIXED model with cage as random effect, and before weaning (BW: C, D) and after weaning (AW: CC, DC, DD), and their interaction (BW × AW) as fixed effect. As for sensory analysis, the ANOVA MIXED model considered the four panellists as random effect. Flavour perception data were analysed by one-way ANOVA (PROC GLM) with the treatment (C-CC, C-DC, C-DD, D-CC, D-DC, D-DD) as fixed effect. Least square means were obtained using Bonferroni test.

4.5 Experiment 3: *Silybum marianum* meat quality

4.5.1 HL and LTL pHu, colour, thawing and cooking losses

The right HL was deboned and the meat to bone ratio was calculated (Blasco and Ouhayoun, 1996). L*a*b* colour measurements (CIE, 1976) were carried out on the right LTL muscle (RM200QC colorimeter, X-Rite, Co., Neu-Isenburg, Germany). Ultimate pH (pHu at 24 h *post mortem*) was measured in the right LTL meat and Biceps femoris muscle of the right HL, using a portable pH-meter (FG2-Five Go™ Mettler

Toledo, Greifensee, Switzerland). The pHu as well as the colour values represented the average of two repeated measurements.

Right LTL were then vacuum-packed and stored at −40 °C until sensory analysis.

Frozen left HL were allowed to thaw overnight at +4 °C, and subsequently used for thawing and cooking loss determinations. After weighing, HL samples were individually vacuum-sealed using a CSV-41n ORVED machine (99% vacuum level) in polyethylene bags (water vapour transmission rate: 3.5 ± 1 g/m^2 day at 23 °C and 85 ± 2% R.H.), and cooked in a water bath at 80 °C for 1 h. Afterwards, samples were cooled, dried and weighed.

4.5.2 Chemical analyses

The analyses of SM as well as those of the experimental diets were carried out in duplicate using the AOAC (2000) methods to determine the concentrations of dry matter (DM; Method no. 934.01), crude protein (CP; Method no. 2001.11), crude fibre (CF; Method no. 978.10), ash (Method no. 967.05) and starch (amyloglucosidase-α-amylase method, 996.11). Ether extract was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF, without sodium sulphite), acid detergent fibre (ADF), and acid detergent lignin

(ADL) were analysed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest et al. (1991), respectively, using the sequential procedure and the filter bag system (Ankom Technology, New York). The gross energy (GE) was measured with an adiabatic

bomb calorimeter (ISO, 1998). The mineral profile (Ca, P, K, Mg, Na, S, Fe, Zn) of the diets was analysed by ICP-OES (Spectro Ciros Vision

EOP) after microwave digestion (AOAC, 2000, 999.10). The dietary content of vitamins E, B1 and B2 was analysed by EPTA NORD srl (via Padova, Conselve, Italy, internal methods n. PP 475 rev 4 2016, MI 234 rev 1 2014 and MI 235 rev 1 2014, respectively).

4.5.3 Measurement of lipid oxidation

After two months of storage, the left LTL (n=10 samples/treatment) were allowed to thaw for 24 h at +4 °C. They were then individually ground using a Retsch Grindomix GM 200 (7000 g for 10 s). The extent of muscle lipid oxidation was evaluated with a spectrophotometer (Hitachi U-2000, Theodor-Heuss-Anlage 12, Mannheim, F.R. Germany) set at 532 nm, that measured the absorbance of thiobarbituric acid-reactive substances (TBARS) and a 1,1,3,3-tetraethoxypropane calibration curve (Botsoglou et al., 1994). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

4.5.4 Sensory analysis

After 2 months of frozen storage, the 45 right LTL samples (15 per treatment) were subjected to a ranking sensory analysis, conducted by a four-member trained panel belonging to the MAPS Department (Italy). In order to familiarise with the food matrix and to select the appropriate descriptors, panel members underwent four pre-test training sessions, testing one rabbit loin/panellist/training session, purchased in a local supermarket. During the last two training sessions, panellists were also trained to familiarise with the ranking test and with the perception of dried ground *Silybum marianum* which was bought in a herbalist's shop.

The test was carried out on three consecutive days: on each day of analysis, 15 samples were evaluated (5 samples×3 treatments) after thawing for 24 h at +4 °C. Vacuum-sealed samples (3 per PVC bag) were identified by a random three-digit code (C, SM1, SM2) and cooked in a water bath at 85 °C until core temperature reached 74 °C.

Each cooked sample (still warm) was cut into four pieces of the same size and assigned to a panellist for the evaluation of sensory attributes. Each descriptor of the meat (rancid odour, herbaceous odour, rabbit odour, rancid flavour, herbaceous flavour and rabbit flavour) was ranked from the least (rank 1) to the most intense (rank 3).

4.5.5 Statistical analysis

Individual records of body weight, average weight gain and carcass traits were evaluated by one-way ANOVA of the statistical analysis software SAS, 2008, version 9.1.3) and processed choosing a mixed model that considered cage as random effect and treatment as fixed effect (PROC MIXED). FI and FCR data, calculated at cage level, were processed with a one-way ANOVA with the treatment as fixed effect (PROC GLM). Meat quality, TBARS and sensory analysis were processed with another one-way ANOVA with the treatment as fixed effect.

A Chi-squared test with the Marascuilo (1966) procedure was performed on mortality data to detect the differences among the treatments. Bonferroni adjustments and three significance levels were assigned: *: $P < 0.05$; **: $P < 0.01$; ***; $P < 0.001$.

5. DIETARY SUPPLEMENTATION OF DIGESTAROM® HERBAL FORMULATION: EFFECT ON APPARENT DIGESTIBILITY, FAECAL AND CAECAL MICROBIAL COUNTS AND LIVE PERFORMANCE OF GROWING RABBITS

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DIETARY SUPPLEMENTATION OF DIGESTAROM® HERBAL FORMULATION: EFFECT ON APPARENT DIGESTIBILITY, FAECAL AND CAECAL MICROBIAL COUNTS AND LIVE PERFORMANCE OF GROWING RABBITS

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Abstract: The experiment aimed to study the effect of Digestarom® dietary inclusion (herbal formulation containing a mixture of essential oils, herbs, spices and extracts) on apparent digestibility and digestive ecosystem of growing rabbits, as well as the effects of its supplementation before and after weaning on growth performance. At kindling, rabbit does and litters were divided into 2 dietary groups (51 does/group) and fed either a control diet (C) or a diet supplemented with 300 mg Digestarom®/kg diet (D) until weaning, which occurred at 35 d (before weaning supplementation). Each group was further divided into 3 dietary groups: CC received the control diet and DD received the D diet from 5 to 12 wk of age, and DC were fed with D (from 5 to 8 wk of age) and C diets (from 8 to 12 wk of age) (after weaning supplementation; 54 kits/group). An *in vivo* digestibility trial and a faecal microbial count were carried out on growing rabbits that received only the C or D diets during the trial. The C group showed higher DM intake than D group (215 *vs.* 196 g/d; $P<0.05$). The faecal digestibility of ether extract (75.9 *vs.* 59.8%; $P<0.001$), cellulose (25.9 *vs.* 20.6%; $P<0.05$) and gross energy (51.8 *vs.* 49.1%; $P<0.05$) was higher for C than for D group, whereas that of starch (98.9 *vs.* 98.8%; $P<0.001$) and the digestible protein to digestible energy ratio (13.9 *vs.* 13.2 g digestible protein/MJ digestible energy; $P<0.01$) was the highest for rabbits fed D diet. Stomach and caecal pH, caecal and faecal microbial counts were independent of the dietary treatment. The only exception was the stomach pH in 8 wk-old rabbits, which had the lowest value in C rabbits ($P<0.05$). The D supplementation before weaning improved feed conversion ratio throughout the growing phase (4.3 *vs.* 4.4 for D and C, respectively; $P<0.05$), whereas significant differences in daily weight gain, feed conversion ratio and mortality were observed only in the first period after weaning. Based on the results obtained, dietary supplementation with Digestarom® does not seem to confirm the positive results previously reported for growing rabbits.

Key Words: rabbit, Digestarom®, faecal digestibility, microbial count, performance.

INTRODUCTION

Digestarom® 1315 is a herbal formulation designed as rabbit feed supplement, consisting of a mixture of essential oils, herbs, spices and extracts of 10 different ingredients (Colin *et al.,* 2008): onion (*Allium cepa* L.*)*, garlic (*Allium sativum* L.), caraway (*Carum carvi* L.), fennel (*Foenicum vulgarae* L.), gentian (*Gentiana lutea* L.), melissa (*Melissa officinalis* L.), mint (*Mentha arvensis* L.), anise (*Pimpinella anisum* L.), oak bark (*Quercus cortex*) and clove (*Syzygium aromaticum* L.).

Studies on single plant extracts constituting Digestarom® feed supplement have reported several positive effects on animal health and live performance. Dehydrated onion, at 5 or 10% inclusion level, showed cholesterol-lowering and antioxidant effects in hyper-cholesterolemic experimental rats (Vidyavati *et al.,* 2010). Histological and biochemical

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studies using suitable dosage of garlic according to the body weight found positive effects of different in-feed garlic extracts on hepatic coccidiosis in infected rabbits (Toulah and Al-Rawi, 2007), as well as cholesterol levels of blood and oxidative status of the hepatic tissue in cholesterol-fed rabbits, treated with 1,5 mL/kg day of garlic extract for 3 mo (Arhan *et al.,* 2009). In addition, dietary fermented garlic demonstrated a beneficial effect on the immune response during an inflammatory challenge in growing pigs, reporting a linear immune response as fermented garlic increased from 1, 2 to 4 g/kg (Wang *et al.,* 2011). Garlic meal positively affected intestinal mucosal morphology of broiler chickens when supplemented at 1 or 2% inclusion level, thus potentially improving nutrients absorption (Adibmoradi *et al.,* 2006). A simultaneous supplementation of garlic (50 g granulate powder) and aniseed (25 g) was reported to improve feed intake in post-weaned piglets (Langendijk *et al.,* 2007).

In growing rabbits, mortality was reduced with the supplementation of 0.05% essential oil of fennel and thyme (Benlemlih *et al.,* 2014). Furthermore, a diet supplemented with 0.5% fennel seed increased the digestibility of organic matter, crude fibre and ether extract, and final weight and body weight gain improved (Omer *et al.,* 2013).

An essential oil of *Melissa officinalis* contributed to a lipid-lowering action in cholesterol-fed rabbits (Karimi *et al.,* 2010), whereas a dietary addition of peppermint improved crude protein digestibility (Ibrahim *et al.,* 2000).

Oak bark is traditionally used for human consumption as a decoction and powder to treat gastrointestinal problems, such as diarrhoea, gastritis and ulcer, and 10 µL of extract impregnated in sterile discs showed antibacterial activity against reference strains *in vitro* (Berahou *et al.,* 2007).

Clove, caraway and gentian were reported to provide appetite-stimulant effect (Baytop, 1984; Loo and Richard, 1992; Wichtl, 1994).

Digestarom® feed additive was tested only in 3 experiments in rabbits, all considering an inclusion level of 300 mg Digestarom®/kg feed. Colin *et al.* (2008) found a reduction in mortality rate in a field trial with 19000 rabbits (13.4 *vs.* 14.2%; $P<0.01$), and Krieg *et al.* (2009) observed a positive effect on the performance and health of weaned rabbits for the 13 days observation period, whereas Abd El-Hady *et al.* (2013) observed an improvement in growth performance, some blood constituents and carcass characteristics of growing rabbits.

The present study aimed to investigate in depth the effects of dietary supplementation of Digestarom® on the total tract apparent digestibility, faecal and caecal microbial counts, live performance and health status of growing rabbits measured at different times during the growing period. For the first time, the effects of before and after weaning supplementation on the live performance of growing rabbits were considered. Reproductive performance scores of rabbit does were also evaluated, but results are presented elsewhere (Celia *et al.,* 2015).

MATERIAL AND METHODS

The study was approved by the Institutional Animal Welfare Committee as the animal-welfare body of the Kaposvár University. All animals were handled according to the principles stated in the EC Directive 86/60963/2010 EU regarding the protection of animals used for experimental and other scientific purposes.

Animals and experimental design

The experiment was carried out in the experimental farm of Kaposvár University. The animals derived from a previous part of the experiment which also aimed to evaluate the effect of dietary supplementation with Digestarom® on the reproductive performance of rabbit does (Celia *et al.,* 2015). At kindling, does and litters were divided in 2 dietary groups and fed with balanced pelleted diets (Table 1): the first group (51 does/group) received a commercial diet (group C), whereas the other one (52 does/group) was fed the same diet supplemented with 300 mg/kg of Digestarom® (group D). However, the litters were fed experimental diets from 21st d of life onward. This represented the Before Weaning phase (BW), described in a previous article (Celia *et al.,* 2015). At weaning (35 d), each group was further divided into 3 feeding groups: CC rabbits received the C diet and DD ones received the D diet from 5 to 12 wk of age. Differently, DC rabbits were fed with D and C diets from 5 to 8 wk of age and from 8 to 12 wk of age, respectively (Figure 1). This represented the After Weaning phase (AW). The experiment involved 372 growing rabbits of the Pannon breeding programme (Pannon Ka maternal line). Among them, 324 rabbits were used to evaluate the growth performance (54 rabbits/diet), whereas 48 rabbits were used

for gastrointestinal pH and caecal microbial count analyses. From the 48 rabbits, 12 were slaughtered at 5 wk of age (6 rabbits/diet), and 36 were reared separately, then 24 were slaughtered at 8 wk of age (6 rabbits/diet). Remaining rabbits were not considered for the study. The kits were housed in wire-mesh cages (3 rabbits/cage, size of cage: 61×32×30 cm). The temperature and the photoperiod were 15-18°C and 16 h light:8 h dark, respectively.

Performance data collection and management

Body weight of rabbits was measured at 5, 8 and 12 wk of age, feed intake for 5-8 and 8-12 wk periods was recorded and the daily weight gain and feed conversion ratio were then calculated. Body weight and daily weight gain were evaluated based on individual data, whereas feed intake and feed conversion ratio were based on the cage unit. When calculating feed intake, it was assumed that morbid rabbits did not consume any pellets for the 2 d preceding their death. Mortality was recorded daily.

pH of the stomach and caecal content and caecal microbial count

From 13:00 to 14:00 h six healthy rabbits per experimental group were slaughtered at 5 (6 C and 6 D) and at 8 wk of age (6 C-C, 6 C-D, 6 D-C, 6 D-D). The digestive tract of each animal was removed immediately and the stomach, small intestine and caecum were separated. The pH values of the stomach and caecal contents were determined using an OP-110, Radelkis pH-meter (Hungary).

Table 1: Chemical composition and mineral profile of the experimental diets (g/kg as fed).

	Experimental diets	
	C	D
Dry matter	905	905
Ash	65	70
Acid insoluble ash	0.9	0.2
Crude protein	158	158
Ether extract	30	30
Starch	123	129
Crude fibre	181	165
Neutral detergent fibre	466	448
Acid detergent fibre	231	223
Acid detergent lignin	60	58
K	7.21	7.33
P	5.93	6.16
Ca	5.77	6.21
Mg	2.55	2.62
Na	1.02	1.44
S	0.59	0.57
Fe	0.09	0.09
Zn	0.08	0.07
Ca/P	0.97	1.01
Gross energy (MJ/kg)	16.64	16.50

C: control diet; D: C diet supplemented with 300 mg Digestarom®/kg.

From the 1 g sample taken from the caecal digesta of each rabbit (serial dilutions were made: 1 g caecal sample+9 mL diluent [0.9% NaCl]), and used for microbiological determination. Anaerobic conditions were ensured by the use of carbon dioxide.

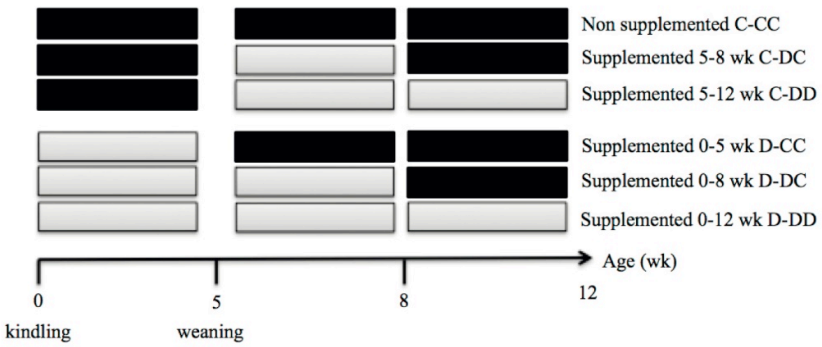

Figure 1: Experimental design (n=54 rabbits/treatment). ▢ Diet D, supplemented with 300 mg Digestarom®. ■ Diet C.

The obligate anaerobe microorganisms were cultured on Schaedler's agar (Sharlan Chemie, Barcelona, Spain), the selectivity of which was increased by the addition of esculin (Merck, Darmstadt, Germany), neomycin (Merck, Darmstadt, Germany) and iron ammonium citrate (Sharlan Chemie, Barcelona, Spain). Gamma sterile Petri dishes (Biolab, Budapest) were placed into Anaerocult culture system (Merck, Darmstadt, Germany), in which the anaerobic conditions were ensured by an "Anaerocult A" (Merck, Darmstadt, Germany) gas-producing bag. Subsequently, the samples were incubated in an LP 104 type thermostat (LMIM, Esztergom, Hungary) at 37°C for 96 h.

Total aerobic bacteria were cultured on media supplemented with 5% calf blood. The samples were incubated at 37°C for 72 h. *E. coli* and other *coliform bacteria* were cultured on a Chromocult differentiation medium (Merck, Darmstadt, Germany). The samples were incubated at 37°C, under aerobic conditions, for 24 h.

After the incubation time had elapsed, the colonies were counted according to standard methodology (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem). The colony counts were expressed in $\log_{10}$ colony-forming units (CFU) related to 1 g of sample.

Digestibility trial

An *in vivo* digestibility trial was carried out according to the European standardised method (Perez *et al.*, 1995). To this end, twenty 50 d-old growing rabbits were used to determine the total tract apparent digestibility (TTAD) of C and D diets (10 rabbits/diet). These rabbits received the C or D diets during the digestibility trial, only. Animals were equally distributed by gender and live weight (average live weight of 1478±142 g) into the 2 dietary groups and individually caged. After 1 wk of adaptation to the new diets, faeces were collected for a 4-d period. Morbid and/or dead rabbits were excluded from the trial; they were not replaced and not considered in the statistical analysis.

The TTAD of dry matter (DM), organic matter, crude protein, ether extract, starch, neutral detergent fibre, acid detergent fibre, cellulose, hemicelluloses and gross energy of the experimental diets (C and D) was measured.

The day after the end of the digestibility trial, the rabbits continued to be fed the same experimental diets. Samples of hard faeces were collected from each animal and immediately submitted to the quantitative determination of coliforms, lactic acid bacteria and spore-forming aerobes (*Bacillus* spp.). Coliforms were counted using the same procedure previously reported for caecal content. The lactic acid bacteria load was measured by plating on MRS agar (Scharlan Chemie, Barcelona, Spain) after anaerobic incubation at 37°C for 48 h. The count of spore-forming *Bacillus* spp. was determined by plating on Bacillus Selective Agar (Oxoid LTD, Basingstoke, Hampshire, England) after aerobic incubation at 37°C for 24 h. Colony counts were expressed in $\log_{10}$ CFU related to 1 g of sample.

Chemical analyses

Chemical composition of the experimental diets and faeces was analysed in duplicate by AOAC (2000) methods to determine the concentrations of dry matter (Method no. 934.01), crude protein (Method no. 2001.11), crude fibre (Method no. 978.10) and ash (Method no. 996.11). Ether extract was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF without sodium sulphite), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to Mertens (2002), AOAC (2000, Method no. 973.187), and Van Soest *et al.* (1991), respectively, using the sequential procedure and filter bag system (Ankom Technology, New York). The gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO, 1998). The mineral profile (Ca, P, K, Mg, Na, S, Fe, Zn) of the experimental diets was analysed by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10).

Statistical analysis

Digestibility data, faecal microbial count during the digestibility trial and caecal microbial count of rabbits at 5 wk of age were analysed by one-way ANOVA of the GLM procedure of the Statistical Analysis System (SAS Institute, 2004). Experimental diets (C, D) were considered as fixed effect. Live performance and caecal microbial count of rabbits at 8 wk of age data were subjected to another ANOVA in which a PROC MIXED procedure tested the effect of dietary supplementation before weaning (BW), after weaning (AW) and their interaction (BW x AW) on the studied variables. Microbial count data were also analysed by one-way ANOVA with age (5 and 8 wk of age) as fixed effect. Mortality

data were analysed by chi-square test according to the Marascuilo (1966) procedure. Post hoc pairwise contrasts were evaluated by Bonferroni adjustments.

RESULTS AND DISCUSSION

Digestibility trial and faecal microbial count

Dry matter intake (DM, g) as well as DM intake/live weight (g/kg LW) were higher in C compared to D rabbits ($P<0.05$. Table 2). The TTAD of cellulose was higher in C than D diet ($P<0.05$), as was that of ether extract ($P<0.001$), the latter explaining the better energy digestibility ($P<0.05$) and nutritive value of the C diet in terms of digestible energy (DE, MJ/kg; $P<0.05$). Conversely, digestible protein (DP) to DE ratio was in favour of D diet ($P<0.01$). Also starch TTAD was higher in D diet ($P<0.001$). The DE of both dietary treatments was in the normal range recommended for growing rabbits, but under 10-10.5 MJ/kg, which ensures maximum average daily growth (Xiccato and Trocino, 2010).

Results from this study found partial confirmation in the work considering the digestibility coefficients of 63 d-old Alexandria rabbits supplemented with 300 and 400 mg Digestarom®/kg of feed (Abd El-Hady *et al.,* 2013). In fact, crude fibre digestibility worsened as Digestarom® supplementation increased. However, organic matter digestibility was the best in supplemented animals, whereas no effect of the dietary treatment on this trait was observed in our experiment. In general, as a probable effect of age, TTAD scores in our experiment tended to be lower than those presented in the work of Abd El-Hady *et al.* (2013), especially when considering the DM (–27%, on av.).

The lower DM intake of D rabbits compared to C ones might be explained by the tannin-like substances included in Digestarom® which could have negatively influenced the palatability of the feed, as was observed in a study testing a dietary supplementation of a tannin extract derived from quebracho trees in growing rabbits, and in another one in which calves' diet was supplemented with a dry pomegranate extract (Dalle Zotte and Cossu, 2009; Oliveira *et al.,* 2010). In fact, tannins are known to form complexes with salivary glycoproteins generating the astringency sensation,

Table 2: Effect of Digestarom® dietary supplementation on total tract apparent digestibility (TTAD) of 50 d-old growing rabbits and nutritive value of diets.

	Experimental diets			
	C	D	MSE	Significance
n	5	7		
Live Weight (g)	2018	1976	72.1	NS
Dry Matter intake (g/d)	215	196	49.0	*
TTAD (%)				
DM	49.9	48.6	1.9	NS
Organic matter	50.5	48.6	1.9	NS
Crude protein	71.9	71.4	1.1	NS
Ether extract	75.9	59.8	1.4	***
Starch	98.8	98.9	0.04	***
Neutral detergent fibre (NDF)	28.6	26.7	2.7	NS
Acid detergent fibre (ADF)	16.6	13.9	3.2	NS
Cellulose (ADF-Acid detergent lignin)	25.9	20.6	2.9	*
Hemicelluloses (NDF-ADF)	40.3	39.4	2.3	NS
Gross energy	51.8	49.1	1.9	*
Nutritive value:				
Digestible protein (DP) (g/kg)	125.7	124.6	1.9	NS
Digestible energy (DE) (MJ/kg)	9.52	8.96	0.3	*
DP to DE ratio (g/MJ)	13.21	13.92	0.3	**

C: control diet; D: C diet supplemented with 300 mg Digestarom®/kg; MSE: mean squared error.
Levels of significance: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; NS, non-significant.

Table 3: Effect of Digestarom® dietary supplementation on faecal microbial count during the digestibility trial.

	Experimental diets			
	C	D	MSE	Significance
n	5	7		
Coliforms, log_{10} CFU/g	7.34	8.06	1.02	NS
Lactic acid bacteria, log_{10} CFU/g	5.92	6.19	1.00	NS
Bacillus spp., log_{10} CFU/g	8.54	8.15	0.71	NS

C: control diet; D: C diet supplemented with 300 mg Digestarom®/kg; MSE: mean square error; NS: no significant.

thus reducing feed intake (Gidenne *et al.,* 1998). In contrast, chestnut hydrolysable tannins added as a supplement to growing rabbit diets did not impair the nutritive value of diets (Dalle Zotte *et al.,* 2012).

The negative effect of Digestarom® dietary supplementation on ether extract and cellulose TTAD could be explained by some constituents of plant polyphenols also present in Digestarom®, as they could have inhibited the activity of certain digestive enzymes. In fact, some polyphenol components can inhibit protease activity thus affecting protein digestion, whereas others can exert a lipase-inhibition activity, thus negatively affecting fat digestion (McDougall *et al.,* 2008). In this sense, when considering food producing animals such as rabbits, one of the most challenging aspects concerning natural feed additives is to find the optimum inclusion level that can guarantee satisfactory performance without impairing nutrient digestibility and absorption.

As a confirmation of this potential negative effect of specific components of plant polyphenols on the digestibility of nutrient fractions, Peiretti and Meineri (2008), Dalle Zotte *et al.* (2013) and Gerencsér *et al.* (2014) also observed a negative effect of different levels of spirulina and thyme dietary supplementation in growing rabbits on TTAD of DM, NDF, ADF, crude protein, starch, ether extract and minerals.

Table 3 depicts faecal microbial count of rabbits used for the digestibility trial and fed C or D diets. Even if no statistical differences were found between the 2 dietary groups, an unexpected situation was observed: the quantity of coliforms in the faeces was high in both treatments (7.34 and 8.06 log_{10} CFU/g for C and D faeces, respectively). The flow of caecal matter through the colon could have increased the specific charge of coliforms, leading to the high amount found.

Placha *et al.* (2013) observed that the dietary inclusion of 0.5 g/kg of thyme essential oil was able to limit the colonisation of coliforms in the caecum (<1.0 log_{10} CFU/g), compared to the control diet (2.4 log_{10} CFU/g); however, a higher coliforms content was found in faeces of rabbits fed with the thyme essential oil supplement (4.81 log_{10} CFU/g).

Lactic acid bacteria, which are not considered regular inhabitants of the digestive tract of rabbits by some authors (Gidenne and Fortun-Lamothe, 2002; Combes *et al.,* 2013), were also found in the faeces of both dietary treatments. However, they are reported to positively affect the health status of rabbits, as noted in a study in which *Lactobacillus plantarum* was sprayed on the litters (5 mL/rabbit) in the pre-weaning period (Bovera *et al.,* 2012). In our study, high counts of *Bacillus* spp. were found in rabbits fed both C and D diets. It should be noted that *Bacillus* spp. is a normal member of the rabbit intestinal microflora, as well as *Bacteroides* spp., and these high counts may have a positive effect on regular gut function because they are inducers of gut-associated lymphoid tissue development (Mage *et al.,* 2006; Hanson and Lanning, 2008; Carabaño *et al.,* 2010). High levels of lactic acid bacteria and *Bacillus* spp. could have played a role in preventing the mortality of rabbit after weaning (5-8 wk of age; Table 6).

Gastrointestinal pH and caecal microbial count of 5 and 8 wk-old rabbits

In 5 wk-old rabbits, a dietary supplementation with Digestarom® had no influence on stomach and caecal pH, total anaerobic and aerobic bacteria and counts of *E. coli*, Coliforms and *Bacteroides* (Table 4). An identical situation was observed in 8 wk-old rabbits in which the BW and AW supplementation with Digestarom® did not affect the studied traits (Table 5). The only exception was the pH of the stomach content of AW rabbits, which was higher in D than C dietary group (1.93 *vs.* 1.63, for D and C, respectively; $P<0.05$). However, these values were within the physiological range in accordance with the age (pH=1.5-2.0, Fortun-Lamothe and Gidenne, 2009).

Table 4: Effect of Digestarom® dietary supplementation before weaning on gastrointestinal pH, and caecal microbial count of rabbits at weaning (5 weeks of age).

	Experimental diets			
	C	D	MSE	Significance
n	6	6		
Body weight (g)	908	937	0.05	NS
pH of stomach content	1.38	1.46	0.30	NS
pH of caecal content	6.44	6.36	0.21	NS
Total aerobic bacteria[a]	5.51	5.54	0.58	NS
Total anaerobic bacteria[a]	9.58	9.31	0.22	NS
Escherichia coli[a]	3.44	3.77	1.87	NS
Coliforms[a]	1.90	1.97	0.12	NS
Bacteroides[a]	8.80	8.93	0.40	NS

C: control diet; D: C diet supplemented with 300 mg Digestarom®/kg; MSE: mean squared error; NS: non-significant.
[a]Germ counts expressed in $\log_{10}$ colony-forming units/g caecal content.

When Digestarom® dietary supplementation was tested in 41 d-old ZIKA® hybrid rabbits, reduced bacterial diversity in the caecum and increased relative abundance of the more dominant species compared to rabbits fed with a non-supplemented diet were observed (Krieg *et al.,* 2009). In addition, Abd-El-Hady (2014) also found that a dietary supplementation with Digestarom® reduced the caecal microbial count of total bacteria, as well as those of *Clostridium* spp. and *E. coli.* The latter study, however showed a higher count for *E. coli* (6.09 $\log_{10}$ CFU/mL caecal content for 63 d-old rabbits, on av.) compared to that in our study (*E. coli*: 3.21 $\log_{10}$ CFU/g caecal content for 8 wk-old rabbits, on av.).

Increasing rabbit age from 5 to 8 wk resulted in a proportional lower density of anaerobic bacteria (9.45 *vs* 8.22 $\log_{10}$ CFU/g; (P<0.001), as well as *Bacteroides* (8.86 *vs.* 7.84 $\log_{10}$ CFU/g; P<0.001). In an experiment testing the effect of spirulina and thyme dietary supplementation on digesta traits and caecal microbiota, Bónai *et al.* (2012) observed the same decreasing trend of total anaerobic bacteria with increasing age of rabbits. The higher (P<0.01) stomach pH of 8 week-old rabbits compared to 5 week-old ones (1.78 *vs.*1.42) was also in agreement with the above mentioned study and within the normal range reported in the literature (Gidenne and Lebas, 2005).

Table 5: Effect of Digestarom® dietary supplementation on body weight, gastrointestinal pH, and caecal microbial count of growing rabbits (8 weeks of age).

	Experimental diets					Significance of diet		
	Before weaning (BW)		After weaning (AW)					
	C	D	C	D	MSE	BW	AW	BW×AW
n	6	6	6	6				
Body weight (g)	1858	1803	1824	1837	110	NS	NS	NS
pH of stomach content	1.72	1.85	1.63	1.93	0.33	NS	*	NS
pH of caecal content	6.12	6.24	6.25	6.11	0.16	NS	NS	NS
Total aerobic bacteria[a]	5.38	5.54	5.47	5.44	0.23	NS	NS	NS
Total anaerobic bacteria[a]	8.28	8.17	8.26	8.19	0.50	NS	NS	NS
Escherichia coli[a]	3.20	3.30	3.12	3.39	1.66	NS	NS	NS
Coliforms[a]	2.13	2.22	2.33	2.01	0.66	NS	NS	NS
Bacteroides[2]	7.75	7.93	7.86	7.82	0.52	NS	NS	NS

C: control diet; D: C diet supplemented with 300 mg Digestarom®/kg; MSE: mean squared error.
Level of significance: *: P<0.05; NS: non-significant.
[a]Germ counts expressed in $\log_{10}$ colony-forming units/g caecal content.

Table 6: Effect of Digestarom® dietary supplementation on live performance of growing rabbits.

	Experimental diets								
	Before weaning (BW)		After weaning (AW)				Significance		
	C	D	CC	DC	DD	MSE	BW	AW	BW×AW
n	162	162	108	108	108				
Body Weight (g)									
5 wk	887	880	881	883	886	6.9	NS	NS	NS
8 wk	1776	1784	1743	1802	1795	10.6	NS	NS	NS
9 wk	2019	2013	1993	2022	2032	12.5	NS	NS	NS
12 wk	2643	2663	2645	2654	2659	15.9	NS	NS	NS
Average weight gain (g/d)									
5-8 wk	42.3	43.0	41.0^{a}	43.8^{b}	43.3^{b}	0.3	NS	**	NS
8-12 wk	30.8	31.3	32.1	30.7	30.4	0.4	NS	NS	NS
5-12 wk	35.8	36.4	36.0	36.3	36.0	0.3	NS	NS	NS
Feed intake (g/d)									
5-8 wk	130.5	128.4	130.1	129.7	128.6	1.0	NS	NS	NS
8-12 wk	176.0	174.5	179.2	172.7	173.8	1.8	NS	NS	NS
5-12 wk	156.5	154.8	158.1	154.3	154.5	1.2	NS	NS	NS
Feed conversion ratio									
5-8 wk	3.1	3.0	3.2^{b}	3.0^{a}	3.0^{a}	0.0	*	***	NS
8-12 wk	6.0	5.8	5.9	6.0	5.8	0.1	NS	NS	NS
5-12 wk	4.4	4.3	4.4^{b}	4.3^{ab}	4.3^{a}	0.0	*	*	NS
Mortality (%)									
5-8 wk	0.0	1.9	2.8^{b}	0.0^{a}	0.0^{a}	-	NS	*	-
8-12 wk	8.6	8.0	10.2	6.5	8.3	-	NS	NS	-
5-12 wk	8.6	9.9	13.0	6.5	8.3	-	NS	NS	-

C and CC: control diet; D and DD: C diet supplemented with 300 mg Digestarom®/kg; DC: between 5 and 8 wk D diet and between 8 and 12 wk C diet; MSE: mean squared error.

Level of significance: *: $P<0.05$; **: $P<0.01$; *: $P<0.001$; NS: non-significant.

^{ab}Means in the same row with different superscript letters are significantly different ($P<0.05$).

Live performance depending on Digestarom® supplementation before and after weaning

Digestarom® dietary supplementation had a positive effect on feed conversion ratio from 5 to 8 and from 5 to 12 wk of age ($P<0.05$), whereas it did not affect the body weight, average daily weight gain, feed intake and mortality of the rabbits (Table 6). Although TTAD of some nutrients was lower in 50 d-old Digestarom®-fed rabbits compared to the C group, no negative effects on live performance were observed. Average daily weight gain improved when rabbits consumed the D diet from 5 to 8 wk of age ($P<0.01$). Consequently, feed conversion ratio was also better in DC and DD animals compared to CC ones ($P<0.001$). Moreover, supplementation with Digestarom® during AW did not show mortality from 5 to 8 wk of age, which is a good outcome in the most critical phase of growing rabbits (Rashwan and Marai, 2000). No interaction between before and after weaning supplementation was observed for growth traits.

Even if no studies have tested the Digestarom® dietary supplementation in both BW and AW periods, the literature reports few studies where Digestarom® has been tested on live performance, exhibiting results not always comparable to the present experiment. In 41 d-old rabbits, Krieg *et al.* (2009) observed a higher daily weight gain, daily feed intake and higher final body weight in Digestarom® fed rabbits (300 mg/kg diet) compared to the control group. In addition, Digestarom®-supplemented rabbits had fewer digestive disorders. Similarly, Abd-El-Hady *et al.* (2013) and Abd-El-Hady (2014) found higher final body weight and better feed conversion ratio in Digestarom®-supplemented rabbits (300 mg/kg diet) than those fed with a control diet (from 4 to 9 wk of supplementation in both experiments). Moreover, Colin *et al.* (2008) showed an improved feed conversion ratio and lower mortality in rabbits fed with Digestarom® (300 mg/kg diet) compared to the untreated ones.

The positive results on the live performance of growing rabbits observed in the studies testing Digestarom® dietary supplementation were generally attributed to the substantial reduction in digestive disorders of farmed rabbits. In fact, the phenolic components of essential oils possess antimicrobial activity against several microorganisms by altering the permeability of the cytoplasmic membrane to hydrogen ions (H^+) and potassium (K^+), leading to the disruption of essential cellular processes (Costa *et al.,* 2013). Chemically, essential oils are complex mixtures of several different components such as terpenoids and many low molecular weight aliphatic hydrocarbons, which often make it difficult to explain their activities (Brenes and Roura, 2010). A work by Stein and Kil (2006) on weanling pigs showed that the hydrophobic constituents of essential oils allowed them to disintegrate the outer membrane of *E. coli* and *Salmonella*, thus inactivating these pathogens. A reduction in the number of pathogenic bacteria would thus change the microbial ecology in favour of beneficial species (Michiels *et al.,* 2009).

However, when essential oils are added to animal diets, results can vary greatly and the reason could be attributed to differences in the type and dose of the essential oils used (Li *et al.,* 2012). In animals with a well-developed sense of smell, for example, if the dose used is too high, the strong smell and/or taste can negatively affect feed intake, thus compromising live performance. Digestarom® had a medium-term negative influence on the reproductive performance of rabbit does and a negative effect of smell on feed palatability was hypothesised to explain these results (Celia *et al.,* 2015). Another important aspect which could strongly affect final outcomes is the stability of essential oils during pelleting, as Maenner *et al.* (2011) showed a substantial loss of activity when essential oils were pelleted at a temperature of 58°C.

CONCLUSION

The inclusion of 300 mg/kg of Digestarom® in a diet for growing rabbits was mainly effective when administered after weaning (from 5 to 8 wk of age), as it was able to increase the growth rate, improve feed efficiency and reduce mortality rate. When considering the whole growing period, Digestarom® supplement had no effect either on the live performance of rabbits or on the microbial counts of the caecal and faecal content, whereas it impaired nutrient digestibility. On the whole, this study did not provide convincing evidence of the efficacy of the Digestarom® dietary supplement.

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6. EFFECT OF PRE- AND POST-WEANING DIETARY SUPPLEMENTATION WITH DIGESTAROM® HERBAL FORMULATION ON RABBIT CARCASS TRAITS AND MEAT QUALITY

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Effect of pre- and post-weaning dietary supplementation with Digestarom® herbal formulation on rabbit carcass traits and meat quality

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ABSTRACT

This study evaluated effects of Digestarom® (D) dietary inclusion before weaning (0–5 weeks old; BW) and/or after weaning (5–12 weeks old; AW) on growing rabbit carcass traits and meat quality. During BW, Pannon-Ka rabbits (does, kits) received two diets: a control diet (C) and one supplemented with 300 mg Digestarom®/kg (D). At weaning, each group was divided into 3 dietary sub-groups: CC and DD received C and D diets from 5 to 12 weeks of age, whereas DC was fed D from 5 to 8 weeks and C from 8 to 12 weeks of age (54 rabbits/group; AW). Rabbits were slaughtered at 12 weeks of age. Digestarom® supplementation improved carcass yield and body mid part proportion only when administered BW. Rabbits fed D BW had higher hind leg meat cooking losses. Loin meat spiciness and rancidity increased with D both BW and AW. In conclusion, Digestarom® herbal formulation was ineffective in improving growing rabbit carcass traits or meat quality.

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1. Introduction

The demand from well-educated consumers for safer and more natural products prompted the EU to definitely ban antibiotics from animal nutrition as feed additives in 2006 for the purpose of precluding a probable presence of residues in the meat and reducing the risk of antibiotic resistance. This has obliged researchers, farmers, and meat processing companies to face the challenge of finding the best alternative solution in obtaining healthy, high-value products.

Many alternatives, such as probiotics, prebiotics, enzymes, organic acids, herbs, spices, and their extracts have been tested in rabbits and other species as feed additives to increase productivity and health (Falcão-e-Cunha, Castro-Solla, Maertens, Marounek, Pinheiro, et al., 2007; Hashemi, Zulkifili, Hair Bejo, Farida, & Somchit, 2008). Plants have been used for centuries around the world as traditional medical remedies, flavour and aroma enhancers, and most recently as food preservers. The healthful effects of several herbs and spices are probably related to their phytochemicals, a wide group of secondary natural compounds considered not essential for the plant's basic function but assumed to play a protection role (Hashemi & Davoodi, 2011).

Digestarom® 1315 is a herbal formulation of a mixture of 10 different herbs and spices designed for broiler rabbits. Digestarom® 1315 contains onion (*Allium cepa* L.), garlic (*Allium sativum* L.), caraway (*Carum carvi* L.), fennel (*Foenicum vulgare* L.), gentian (*Gentiana lutea* L.), melissa (*Melissa officinalis* L.), mint (*Mentha arvensis* L.), anise (*Pimpinella anisum* L.) oak bark (*Quercus cortex*), and clove (*Syzygium aromaticum* L.), many of which are rich in phytochemicals such as flavonoids and carotenoids (Colin, Atkarl, & Prigent, 2008).

Many herbs and spices contain active components capable of exerting antioxidant action. In an *in vitro* study that tested the antioxidant activity of 26 different spices, Shan, Cai, Sun., & Corke (2005) found that clove has the highest total antioxidant capacity (TEAC) (168 mmol/100 g of dry weight). Also essential oil from chamomile (*Matricaria chamomilla* L.) and fennel (*Foeniculum vulgare* L.) exhibited *in vitro* antioxidant activity, in addition to considerable antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, and Salmonella typhi*, and against a yeast, *Candida albicans*, and a mould: *Aspergillus flavus* (Roby, Sarhan, Selim, & Khalel, 2013).

Recent *in vivo* studies have confirmed the antioxidant action of certain herbs and spices, such as dietary supplementation with dried *M. officinalis*, which was found to reduce lipid oxidation in chicken breast and thigh (Kasapidou, Giannenas, Mitlianga, Bouloumpasi, Petrotos, et al., 2014).

In chicken broilers, dietary supplementation of 1% (Raeesi, Hoseini-Aliabad, Roofchaee, Zare Shahneh & Pirali, 2010) or 4% (Kim, Jin & Yang, 2009) garlic powder resulted in higher carcass and breast yield while improving meat texture and flavour. Other benefits of the dietary inclusion of herbs and spices in chickens were reported for fresh onion (3% inclusion level) (Goodarzi, Nanekarani & Landy, 2014), whereas a blend of

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clove and cinnamon essential oil (100 ppm) (Isabel & Santos, 2009) led to higher final body weight and breast yield.

In rabbits, the only study that tested the effect of Digestarom® commercial product on meat quality highlighted an increase in protein and lipid content ($P < 0.05$) in the meat of rabbits fed 300 mg Digestarom®/kg diet, which was most likely due to the animals' increased growth rate (Abd-El-Hady, 2014). Other than this, few studies have considered the effect of dietary inclusion of the herbs and spices present in Digestarom® on carcass and meat quality. In particular, some positive results were obtained by Omer, EL-Nomeary, El-Kady, Badr, Ali, et al. (2013), who observed an improvement in final live weight and body weight gain without any difference in meat proximate composition however when the rabbits' diet was supplemented with 1% fennel seed. No research on growing rabbits available in literature has yet considered the effect of dietary inclusion of the herbs and spices included in Digestarom® on meat sensory traits.

This study evaluated the effect on carcass traits and rheological and sensory meat quality produced by including Digestarom® in the feed given to growing rabbits.

The results presented in this article are part of a wider study on rabbit doe reproductive performance (Celia, Cullere, Gerencsér, Matics, Dalle Zotte, et al., 2015), live performance, health status, apparent digestibility of the diets, and microbial diversity in the caecum and faeces of growing rabbits (Celia, Cullere, Gerencsér, Matics, Giaccone, et al., 2016).

2. Materials and methods

2.1. Animals and experimental design

Maternal line rabbits of the Pannon breeding programme (maternal line: Pannon Ka) were used in this study. At kindling, rabbit does and litters (9–10 kits/litter) were divided into two dietary groups ($n = 162$ kits/dietary group) and fed either a control diet (C) or the C diet (crude protein: 158 g/kg, ether extract: 30 g/kg, starch: 123 g/kg, crude fibre: 181 g/kg) supplemented with Digestarom® (D: 300 mg/kg) herbal formulation. At weaning, which occurred at 35 days of age, both dietary groups were further divided into 3 dietary groups: CC received the C diet and DD the D diet from 5 to 12 weeks of age. Differently, the DC dietary group was fed D and C diets from 5 to 8 weeks of age and from 8 to 12 weeks of age. Overall, 6 feeding groups (54 rabbits/group) were created: C-CC, C-DC, C-DD, D-CC, D-DC, and D-DD (Fig. 1). The animals were housed (3 rabbits/cage) in wire-mesh cages (61 × 32 cm); the temperature and photoperiod were 15–18 °C and 16 L: 8D, respectively.

2.2. Slaughtering, carcass dissection and meat sampling

At 12 weeks of age, rabbits were transported to a slaughterhouse located 200 km from the experimental farm. After fasting (6 h, inclusive of 4 h for transportation) and electro-stunning, rabbits were slaughtered by cutting the carotid arteries and jugular veins. Carcasses were dissected according to World Rabbit Science Association (WRSA) recommendations as described by Blasco & Ouhayoun (1996). The slaughtered rabbits were bled, and then the skin, genitals, urinary bladder, gastrointestinal tract, and the distal part of legs were removed. Warm carcasses (with head, set of organs consisting of the thymus, trachea, oesophagus, lung, and heart, liver, kidneys, and perirenal fat and scapular fat) were weighed and the ratio to slaughter weight (SW) was calculated. Carcasses were then chilled at +4 °C for 24 h. The chilled carcasses (CC) were then weighed. The head, set of organs, liver, and kidneys were removed from each carcass to obtain the reference carcass (RC), which included the meat, bones, and fat deposits. The carcasses were then cut between the 7th and 8th thoracic vertebra and between the 6th and 7th lumbar vertebra to obtain the fore, mid, and hind parts, which were weighed separately. The ratio of the head, organs, fat deposits, and carcass parts to either CC or RC weights were calculated as required.

Hind legs (HL, right and left) and *Longissimus thoracis et lumborum* (LTL) muscles were dissected from 15 rabbits per dietary treatment ($N = 90$ rabbits) and weighed. They were then individually packed in polyethylene bags (water vapour transmission rate: 3.5 ± 1 g/m^2·day at 23 °C and 85 ± 2% R.H.), *vacuum*-sealed using a CSV-41n ORVED machine (99% vacuum level), and ice-cooled in portable refrigerators. The next day, samples were transported to the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy) for meat quality analyses. During transport, the temperature of the samples was kept at 4 ± 1 °C. The samples arrived at the MAPS Department around 33 h *post-mortem* and stored in a professional ventilated refrigerator at 4 ± 1 °C. The only exceptions were the right LTL and right HL, which were immediately stored at −40 °C until further analyses.

2.3. HL and LTL pH, colour, thawing and cooking losses, shear force values, and bone traits

Raw left LTL and HL pH was measured 48 h *post-mortem* using a Mettler Toledo FE20 pH-metre at the 5th lumbar vertebra and at the *Biceps femoris* level. Colour values of lightness, redness, yellowness, chroma and hue (CIE, 1976; L*, a*, b*, C* and H°, respectively) were subsequently measured on the same portions using a RM200QC colorimeter (X-Rite, Co, Neu-Isenburg, Germany. Measuring Area: 8 mm; Measuring Geometrics: 45/0 Image Capture; Illuminant/Observer: D65/10). The values adopted are the average of two measurements for each sample. Raw left LTL and HL were then individually packed in polyethylene bags, vacuum-sealed, and stored at −40 °C.

Right LTL and HL meat samples were allowed to thaw overnight at +4 °C, removed from plastic bags, weighed, and subsequently used for thawing and cooking loss determinations. For this purpose, LTL and HL samples were individually *vacuum*-packed in PVC bags and cooked in a water bath at 80 °C for 1 h and at 85 °C for 2.5 h, respectively. Shear force was assessed with a TA-HDi Texture Analyzer (Stable Micro System,

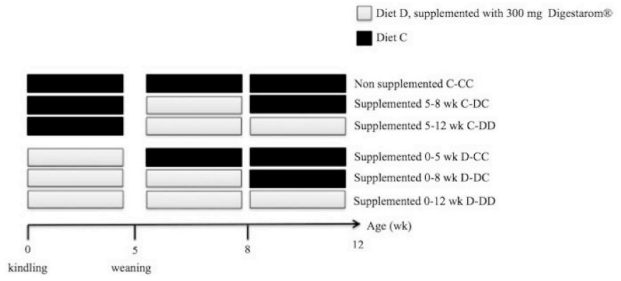

Fig. 1. Experimental design ($n = 54$ rabbits/treatment).

London, UK) on six cylinder-shaped cooked right HL meat pieces per sample (Ø 1.25 cm) sliced perpendicularly to the fibre direction by a Warner-Bratzler cell (100-kg load cell, 2 mm/s crosshead speed) inserted in the texturometer. The WBSF values of each sample are an average of the 6 measurements.

Left HL were thawed under the same procedure used for right HL, and deboned in order to determine the meat/bone ratio (Blasco & Ouhayoun, 1996). Femur and tibia were separately weighed, and then length and minor diameter were measured with a digital calliper (JUWEL *Digital-Schieblehre Rostfrei* H4215/5X A12) before their incidences on HL were calculated. Femur fracture toughness (FT) was calculated at the average bone length point, corresponding to the mid-diaphysis, using a dynamometer Texture TA-HD (SMS- *Stable Micro System*) with a 6 cm wide cell and a load rate of 0.5 mm/s.

2.4. Sensory analysis

After 2 months of frozen storage at −40 °C, the 90 left LTL (15 LTL per treatment) were subjected to a ranking test conducted by a trained four-member MAPS Department panel.

In order to familiarize with the food matrix and to select the appropriate descriptors, panel members underwent four pre-test training sessions, testing one rabbit loin/panellist/training session, purchased in a local supermarket. During the last two training sessions, panellists were also trained to familiarize with the ranking test and with the perception of the herbs and spices constituting Digestarom®, which were bought in a herbalist shop.

The test was carried out in 3 consecutive days in which 30 samples/day were evaluated (5 samples × 6 treatments). Samples were identified by a random three-digit code, *vacuum*-sealed by 6 in PVC bags (D-CC, D-DC, D-DD, C-CC, C-DC, C-DD) and cooked in a water bath at 84 °C until core temperature reached 74 °C every day of sensory analysis after thawing for 16 h at +4 °C. Each cooked meat sample was cut into four numbered pieces of equal size designated to a specific panellist and served still warm for the evaluation of sensory attributes. For each descriptor (olfactory rancidity, olfactory spicy, flavour rancidity, flavour spicy, overall acceptability), meat samples were ranked from least (rank 1) to most intense (rank 6). Lastly, the panellists were also asked which of the ingredients in Digestarom® (onion, garlic, caraway, fennel, gentian, melissa, mint, anise, clove and oak bark) they could recognize (if any).

2.5. Statistical analysis

Data were analysed using SAS 9.1.3 statistical analysis software for Windows (SAS, 2008). Carcass and meat quality were subjected to an ANOVA MIXED model with cage as random effect, and before weaning (BW: C, D) and after weaning (AW: CC, DC, DD), and their interaction (BW × AW) as fixed effect. As for sensory analysis, the ANOVA MIXED model considered the four panellists as random effect. Flavour perception data were analysed by one-way ANOVA (PROC GLM) with the treatment (C-CC, C-DC, C-DD, D-CC, D-DC, D-DD) as fixed effect. Least square means were obtained using Bonferroni test and the significance level was calculated at a 5% confidence level.

3. Results and discussion

3.1. Carcass traits

Dietary D supplementation significantly affected only some carcass traits in either the BW or AW periods, with BW supplementation exerting the greatest effect (Table 1). The reference carcass was heaviest in D rabbits (1279 *vs* 1289 g for C and D rabbits, respectively; $P < 0.001$) and consequently also D rabbit chilled carcass yield was higher (82.3 *vs* 82.8%CC for C and D rabbits, respectively; $P < 0.001$). D supplementation in the BW period provided rabbits with mid parts having a greater incidence on the RC than C rabbits (32.7 *vs* 32.3% for D and C rabbits, respectively). Conversely, results on liver and fore part incidences showed the opposite situation ($P < 0.01$ and $P < 0.05$, respectively).

Differently, AW supplementation did not affect RC weight, even if a growing trend from CC to DD rabbits was observed (1272, 1285, 1295 g for CC, DC and DD rabbits, respectively). The only significant effects of D supplementation in the AW period regarded the HLTTO (heart, lung, thymus, trachea and oesophagus) and kidney incidences on CC ($P < 0.01$), which showed the lowest values in DD animals.

Our results were similar to those of another experiment in which maternal-line rabbits (V-Line) fed 300 mg/kg of Digestarom® from 4 until 9 weeks of age showed higher hot carcass yield and lower incidence of the liver on carcass weight than a control group fed a diet

Table 1
Effect of the dietary Digestarom® supplementation on rabbit carcass traits.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
n of animals	162	162	108	108	108				
Slaughter weight (SW), g	2613	2604	2585	2615	2626	15.24	ns	ns	ns
Chilled Carcass (CC), g	1553	1556	1543	1556	1564	10.3	ns	ns	ns
Reference carcass (RC), g	1279	1289	1272	1285	1295	9.0	***	ns	ns
Chilled carcass yield, % SW	60.9	61.2	61.2	60.9	61.1	0.12	ns	ns	ns
Reference carcass yield, % CC	82.3	82.8	82.3	82.5	82.7	0.07	***	ns	ns
Head, % CC	9.60	9.47	9.55	9.62	9.43	0.04	ns	ns	ns
HLTTO, % CC	1.45	1.42	1.45[a]	1.47[a]	1.38[b]	0.01	ns	**	ns
Liver, % CC	5.15	4.82	5.09	4.88	4.99	0.05	**	ns	ns
Kidneys, % CC	1.14	1.13	1.17[a]	1.14[ab]	1.09[b]	0.01	ns	**	ns
Perirenal fat, % CC	1.11	1.01	1.06	1.02	1.11	0.03	ns	ns	ns
Scapular fat, % CC	0.40	0.37	0.38	0.37	0.40	0.01	ns	ns	ns
Dissectable fat, % CC	1.49	1.37	1.43	1.37	1.50	0.04	ns	ns	ns
Fore part, % RC	28.3	28.0	28.2	28.1	28.2	0.07	*	ns	ns
Mid part, % RC	32.3	32.7	32.4	32.5	32.6	0.08	*	ns	ns
Hind part, % RC	37.6	37.7	37.7	37.7	37.5	0.07	ns	ns	ns
Perirenal fat, % RC	1.35	1.22	1.28	1.23	1.34	0.04	ns	ns	ns

[a, b]Means in the same row having different superscripts are significant at $P \leq 0.05$ level; ns = no significance; MSE = Mean Squared Error; HLTTO = Heart, lung, thymus, trachea and oesophagus.
* $P < 0.05$ level of significance.
** $P < 0.01$ level of significance.
*** $P < 0.001$ level of significance.

Table 2
Effect of the dietary Digestarom® supplementation on hind leg (HL) bones traits.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
n of samples	45	45	30	30	30				
HL bones, g	29.3	29.4	29.0	29.5	29.6	0.31	ns	ns	ns
HL bones, % HL	13.2	13.2	13.2	13.3	13.1	0.13	ns	ns	ns
Femur, g	12.6	12.5	12.3	12.5	12.6	0.13	ns	ns	ns
Femur, %	5.65	5.60	5.63	5.65	5.60	0.06	ns	ns	ns
Femur length, mm	92.9	92.7	92.3	93.2	93.0	0.26	ns	ns	ns
Femur minor Ø, mm	6.67	6.70	6.69	6.64	6.73	0.04	ns	ns	ns
Femur fracture toughness, kg	34.3	35.6	35.0	34.7	35.1	0.43	ns	ns	ns
Tibia, g	7.61	7.50	7.46	7.60	7.62	0.07	ns	ns	ns
Tibia, %	3.41	3.38	3.38	3.43	3.38	0.03	ns	ns	ns
Tibia length, mm	67.5	66.4	65.9[b]	68.0[a]	67.0[ab]	0.30	ns	*	ns
Tibia minor Ø, mm	5.42	5.56	5.56	5.47	5.44	0.04	ns	ns	ns

[a, b] Means in the same row having different superscripts are significant at $P \leq 0.05$ level; ns = no significance; MSE = Mean Squared Error.
* $P < 0.05$ level of significance.

without supplementation (Abd-El-Hady, 2014). The hypothesis of an optimal feed utilization due to improved nutrient digestibility was ventured as a possible explanation for such results, but this was not subsequently supported by Celia, Cullere, Gerencsér, Matics, Giaccone, et al., (2016) however, given that D supplementation negatively affected ether extract, cellulose, and gross energy digestibility. The common findings of all existing research on Digestarom® dietary supplementation in growing rabbits (Abd-El-Hady, 2014; Celia et al., 2016; Colin et al., 2008; Krieg, Vahjen, Awad, Sysel, Kroeger, et al., 2009), regard improved weight gain, feed conversion ratio, and good health status in supplemented *vs* non-supplemented animals. Consequently, Digestarom® may exert positive effect on growth performances and carcass traits by improving carbohydrate digestion and immune system response, together with enhanced thyroid function, which is known to be a big factor in animal production (Hefnawy & Tórtora-Pérez, 2010). Thyroid hormones play a key role in animal body metabolism because they stimulate protein synthesis and increase adipose tissue lipolysis and blood glucose level (Marai, Habeeb & Gad, 2002). The latter hypothesis was confirmed by the results provided by Celia et al. (2016), who showed that despite the fact that D supplementation negatively affected ether extract, cellulose and gross energy digestibility, starch digestibility was the highest in the same group, and there were no differences in the final live weight of the D and control groups. On the basis of the considerations above, also the study by Abd-El-Hady (2014) in which D supplemented rabbits exhibited the highest serum lymphocyte level, T3 (plasma triiodothyroxin), growth hormone, immunoglobulins (IgG), and glucose concentrations seems to confirm our hypothesis.

The inclusion of herbs and spices in animal diets is a very complex topic given that they are composite matrixes, and even when single phytochemicals are tested, dose-dependent effect, genotype, age of the animals, and farming conditions can influence the effectiveness of such supplementations. On one hand in fact, dietary inclusions with tannins derived from red quebracho tree (Dalle Zotte & Cossu, 2009), Spirulina algae and/or thyme leaves (Dalle Zotte, Cullere, Sartori, Dal Bosco, Gerencsér, et al., 2014), chestnut hydrolizable tannins (Dalle Zotte, Matics, Bohatir, Sartori, Gerencsér, et al., 2012) or bioflavonoid hesperidin (Simitzis, Babaliaris, Charismiadou, Papadomichelakis, Goliomytis, et al., 2014) were unable to produce any substantial improvement on rabbit carcass traits. On the other hand, Cardinali, Cullere, Dal Bosco, Mugnai, Castellini, et al. (2014) obtained higher carcass weight and carcass yield in rabbit diets supplemented with oregano and a mix of oregano and rosemary. Similarly, Ashour, Alagawany, Reda, & Abd El-Hack (2014) found that carcass yield and relative organs of rabbits were positively affected by dietary supplementation with a *Yucca schigera* extract.

3.2. Physical analyses and rheological traits

In general, HL bone traits (Table 2) were unaffected by the dietary treatment in either the BW or AW phase. The only exception was tibia length in the AW period, in which the CC group had the shortest tibia and the DC group had the longest ($P < 0.05$), with the DD group exhibiting intermediate length. These results were in accordance with growth performance results presented elsewhere (Celia et al., 2015). Tibia length is, in fact, an indicator of linear growth (Masoud et al., 1986).

The effect of Digestarom® dietary supplementation on rheological traits of rabbit HL and LTL had never been studied before. Some rheological traits of HL meat were significantly affected by treatment only when D was supplemented in the BW phase (Table 3). Unexpectedly, cooking losses were significantly higher in D compared to C meat (15.8 *vs* 13.0% for D and C HL, respectively). This result affected also total water losses,

Table 3
Effect of the dietary Digestarom® supplementation on hind leg (HL) rheological traits.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
n of samples	45	45	30	30	30				
HL weight, g	229	229	229	227	231	1.34	ns	ns	ns
Meat to bones ratio	6.64	6.62	6.63	6.61	6.66	0.07	ns	ns	ns
WBSF, kg/cm^2	2.44	2.54	2.52	2.51	2.43	0.05	ns	ns	ns
Thawing losses, %	0.71	0.78	0.77	0.61	0.83	0.04	ns	ns	ns
Cooking losses, %	13.0	15.8	15.6	14.0	13.5	0.40	***	ns	ns
Total losses, %	13.7	16.5	16.4	14.5	14.4	0.41	***	ns	ns

ns = no significance; MSE = Mean Squared Error; WBSF = Warner Bratzler Shear Force.
*** $P \leq 0.001$ level of significance.

Table 4
Effect of the dietary Digestarom® supplementation on *Longissimus thoracis et lumborum* (LTL) rheological traits.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
n of samples	45	45	30	30	30				
LTL weight, g	72.6	75.6	73.7	74.1	74.5	0.78	ns	ns	ns
Thawing losses, %	5.63	6.87	5.97	6.59	6.19	0.34	ns	ns	ns
Cooking losses, %	28.9	28.7	28.9	28.7	28.8	0.56	ns	ns	ns
Total losses, %	34.6	35.6	34.9	35.3	35.0	0.68	ns	ns	ns

ns = no significance; MSE = Mean Squared Error.

which reflected the same situation described for cooking losses, with D meat showing the greatest values (16.5 *vs* 13.7% for D and C HL, respectively). Despite this, WBSF values did not differ among groups.

Also HL weight and meatiness were not affected by D supplementation in the two feeding periods. The LTL rheological trait results presented in Table 4 showed that unlike as observed in HL, D dietary supplementation in both BW and AW phases did not affect LTL weight or thawing, cooking or total losses. Similarly, also LTL and HL meat pH and L*, a*, b* colour values were unaffected by dietary treatment in both BW and AW periods (Table 5). Cooking processes generate losses of liquid and soluble elements from meat. Heat-induced protein denaturation, in fact, causes less water to be trapped inside protein structures held by capillary forces, in this way causing water loss. In general, the higher the meat's core temperature, the lower its water content, due to increased protein denaturation; otherwise, it is the initial fat content that mostly influences fat loss during cooking (Aaslyng, Bejerholm, Ertbjerg, Bertram, & Andersen, 2003). As fat content increases, in fact, the probability of fat coalescing and then leaking from the product also increases as the mean free distance between fat cells decreases (Cullere, Concollato, & Dalle Zotte, 2013). In the present study, however, HL meat proximate composition was not analysed, and therefore the different cooking losses between C and D HL meat could not be explained in this sense. In another part of the study that considered the productive performances of growing rabbits (Celia et al., 2015), average daily weight gain was positively affected when animals ate the D diet from 5 to 8 weeks of age ($P < 0.01$). In this growth phase, muscle tissue development and thus water presence is higher than fat content (Dalle Zotte, 2002), which is negatively correlated to water holding capacity (Hernández, Oliver, & Blasco, 2000), and this potentially explains our finding regarding cooking losses. From 8 to 12 weeks, however, and considering the overall experimental period as well, the average rabbit weight gain was the same in both groups, thus placing the latter hypothesis in doubt. Although dietary supplementation with *Spirulina platensis* and/or thyme (Dalle Zotte et al., 2014) to growing rabbits did not affect HL and LTL rheological traits, Dal Bosco, Gerencsér, Szendrő, Mugnai, Cullere, et al. (2014) found that the dietary inclusion of thyme in the diet of growing rabbits provided an antioxidant effect on the meat tested during refrigerated storage, and also reduced drip loss.

3.3. Sensory analysis

Digestarom® dietary inclusion in the BW period had a remarkable effect on the sensory characteristics of LTL meat (Table 6). Olfactory rancidity ($P < 0.05$), olfactory spicy ($P < 0.01$), flavour rancidity ($P < 0.001$) and flavour spicy ($P < 0.01$), in fact, were higher in D than C meat. AW supplementation with D seemed to have less effect on the sensory attributes of LTL meat than BW inclusion. Nonetheless, in this case as well, olfactory rancidity ($P < 0.001$) and flavour spicy ($P < 0.05$) were higher in DD than CC rabbits. The only significant interaction observed regarded the flavour rancidity descriptor ($P < 0.01$). In general, considering both supplementation periods, when rabbits' diets were supplemented with Digestarom®, their LTL meat was judged more spicy and more rancid than that of untreated animals. As a consequence of these results, also overall flavour seemed to decrease in animals fed D in both BW and AW periods, with longer supplementation providing the worst results (BW: 3.58 *vs* 3.36 in BW for C and D groups; AW: 3.57 *vs* 3.43 *vs* 3.41 for CC DC and DD groups, respectively), even if the differences were not statistically significant. Panellists clearly perceived a certain spiciness in the meat of rabbits fed D supplements, and even if not enough to affect overall

Table 5
Effect of the dietary Digestarom® supplementation on *Biceps femoris* (BF) and *Longissimus thoracis et lumborum* (LTL) muscles pH and colour values.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
BF muscle									
pH[a]	5.97	5.98	5.99	6.00	5.95	0.02	ns	ns	ns
L*[b]	50.7	50.2	49.7	50.8	50.8	0.39	ns	ns	ns
a*[b]	−2.05	−2.03	−1.91	−2.25	−1.96	0.12	ns	ns	ns
b*[b]	0.55	0.74	0.67	0.41	0.87	0.22	ns	ns	ns
C*	3.13	2.91	2.87	3.01	3.18	0.11	ns	ns	ns
H°	168	160	164	168	159	4.73	ns	ns	ns
LTL muscle									
pH[a]	5.61	5.62	5.60	5.63	5.62	0.01	ns	ns	ns
L*[b]	45.7	46.3	47.0	44.8	46.2	0.69	ns	ns	ns
a*[b]	−2.24	−2.35	−2.37	−2.09	−2.43	0.16	ns	ns	ns
b*[b]	9.06	8.56	8.80	8.93	8.71	0.20	ns	ns	ns
C*	9.60	9.02	9.25	9.36	9.34	0.15	ns	ns	ns
H°	106	105	104	104	108	1.47	ns	ns	ns

ns = no significance; MSE = Mean Squared Error.
[a] pH measured 48 h *post mortem* on BF and LTL muscles of all the slaughtered rabbits.
[b] L*a*b* colour values measured 48 h *post mortem* on BF and LTL muscles of all the slaughtered rabbits.

Table 6
Sensory analysis (ranking test) of Digestarom® *Longissimus thoracis et lumborum* (LTL) muscles.

Periods	Before weaning (BW)		After weaning (AW)			MSE	Significance		
Experimental diets	C	D	CC	DC	DD		BW	AW	BW × AW
n of samples	45	45	30	30	30				
Olfactory rancidity	3.27	3.67	3.00[b]	3.64[a]	3.78[a]	1.66	*	***	ns
Olfactory spicy	3.20	3.75	3.44	3.59	3.4	1.68	**	ns	ns
Flavour rancidity	3.00	3.95	3.26	3.46	3.71	1.61	***	ns	**
Flavour spicy	3.24	3.70	3.45[ab]	3.18[b]	3.78[a]	1.67	**	*	ns
Flavour overall	3.58	3.36	3.57	3.43	3.41	1.70	ns	ns	ns

[a, b] Means in the same row having different superscripts are significant at $P \leq 0.05$ level; ns = no significance; MSE = Mean Squared Error.
* $P < 0.05$ level of significance.
** $P < 0.01$ level of significance.
*** $P \leq 0.001$ level of significance.

flavour perception negatively, it was not appreciated because they associated it with olfactory and flavour rancidity. A similar finding was observed also in a recent study by Cullere, Tasoniero, Contiero, & Dalle Zotte (2015) on rabbit meat treated with increasing levels of rooibos (*Aspalathus linearis*) tea extract as a natural antioxidant. Rancidity and other off-flavours increased with raising rooibos levels, in fact, thus worsening the sensory acceptability of the meat when incorporation percentage exceeded a certain threshold value. The sensory attributes of meat obtained from animals fed natural compounds documented in literature is contrasting: meat of young hybrid pigs fed a plant extract mix (oregano and sweet chestnut) received higher scores for colour, taste and overall liking (Ranucci, Beghelli, Trabalza-Marinucci, Branciari, Forte, et al., 2015).

Fig. 2 shows the panellists' ability to recognize the single ingredients of Digestarom® when evaluating the meat. As expected, onion and garlic were perceived most. Surprisingly enough, they were also detected in the control meat. These two spices in the same genus (*Allium*) contain thiosulfinates, which are volatile sulphur compounds responsible for their characteristic pungent aroma and taste (Lanzotti, 2006). Despite the precautions taken during the sensory analysis, their persistency evidently affected also the flavour of C group meat. Literature reports that the meat of broilers given garlic supplementation received higher flavour scores than those of untreated animals (Kim et al., 2009).

4. Conclusion

In this study, Digestarom® dietary supplementation appeared to be ineffective in improving growing rabbit carcass traits, especially when given after weaning. Furthermore, even without affecting meat tenderness, before weaning supplementation increased hind leg cooking losses. Moreover, despite the fact that overall flavour perception reached the same scores in all groups, panellists recorded higher scores for spiciness and rancidity descriptors in meat of rabbits fed D. On the basis of the considerations above, Digestarom® does not appear to be an effective natural feed additive for the improvement of carcass traits or meat quality in growing rabbits.

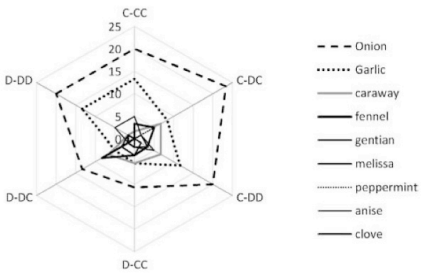

Fig. 2. Flavours perception of Digestarom® spices.

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We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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7. EFFECT OF *SILYBUM MARIANUM* HERB ON THE PRODUCTIVE PERFORMANCE, CARCASS TRAITS AND MEAT QUALITY OF GROWING RABBITS

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Effect of *Silybum marianum* herb on the productive performance, carcass traits and meat quality of growing rabbits

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ABSTRACT

The present study aimed to test the effect of a dietary supplementation with *Silybum marianum* (SM), an herbaceous Mediterranean plant traditionally used to treat liver and gastrointestinal diseases and with antioxidant properties, on the productive performance, carcass traits and meat quality of growing rabbits. With this purpose, at weaning (5 weeks of age), a total of 144 Pannon Large rabbits were allocated to three experimental groups. The control group (C, n=51) was fed with a basal diet, whereas the other groups received the basal diet supplemented with SM herbal powder at two concentrations: 5 g/kg (SM1, n=48) and 10 g/kg (SM2, n=45). Rabbits were housed in wire-mesh cages (3 rabbits/cage) and fed ad libitum throughout the experiment. Productive performance and mortality were recorded weekly. Rabbits were slaughtered at 11 weeks of age, carcasses were dissected, and hind leg (HL) and *Longissimus thoracis et lumborum* (LTL) meat were analysed for meat quality (oxidative status, pHu and L*, a*, b* colour) traits. In addition, a sensory analysis on the LTL meat was carried out by a trained panel. Mortality was significantly reduced in SM treatments compared to C group from week 6–7 (10.4 and 11.1 vs. 17.7%, for SM1, SM2 and C groups, respectively; $P < 0.05$), and in SM2 compared to C and SM1 considering the whole productive cycle (5–11 weeks). The dietary inclusion of SM did not affect carcass traits and did not change neither colour nor oxidative status of LTL muscle. Differently, SM diet increased pHu of LTL muscle (5.98 vs. 6.03 vs. 6.10 in C, SM1 and SM2, respectively; $P < 0.05$). The sensory traits of LTL meat were affected by SM dietary inclusion: a higher herbaceous odour was observed in SM2 compared to C and SM1 ($P < 0.001$) treatments, whereas rabbit odour followed an opposite trend with C receiving a higher score compared to SM1 and SM2 ($P < 0.05$). Panellists also perceived a stronger rabbit flavour in C than in SM1 and SM2 meat (2.40 vs. 1.90 and 1.70, $P < 0.05$; $P < 0.001$). *Silybum marianum* seems to be a promising natural feed additive to improve the health condition of growing rabbits. Differently, the antioxidant activity of *Silybum marianum* was not confirmed when considering fresh meat of rabbits supplemented with the inclusion levels of the present experiment. The dietary supplementation with *Silybum marianum* changed then sensory characteristics of rabbit loin thus, in the future, consumer acceptability should be also carefully assessed.

1. Introduction

The European ban on antimicrobial growth promoters (AGPs) in animal feed, emphasized the necessity to research alternative substances to promote general health and performance in animal production (Barug et al., 2006). Weaning is considered a critical period for the growing rabbit, because procedures such as separation from the mother, changing housing condition, dietary switch from milk to solid feed combined with a non fully-developed immune system, can cause digestive disturbances that influence the growing period (Gidenne et al., 2005; Carabaño et al., 2006; Fortun-Lamothe and Gidenne, 2006; De Blas et al., 2012).

In this context, researchers and feed companies are facing the challenge to fulfil the request of increasingly informed consumers that demand natural and regulated products that simultaneously promote animal health and produce healthy flavourful meat (Dalle Zotte (2002)).

Silybum marianum, popularly known as milk thistle, is an herbaceous plant of the *Asteraceae* family that commonly grows in the Mediterranean countries. *S. marianum* contains mainly flavonoids, a

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group of natural compounds known to have various pharmacological actions such as antioxidant, anti-inflammatory, antitoxic, antibacterial and diuretic (Vogel et al., 1984; Havesteen, 2002; Vaknin et al., 2008; Abed et al., 2015). The major active component of *S. marianum* is silymarin, which includes taxifolin (flavonoid) and seven flavonolignans; silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristrin, and silydianin (Shaker et al., 2010; Colturato et al., 2012). Even though the active component is found in the whole plant, the fruit and the seeds have the highest content (Flora et al., 1998; Vaknin et al., 2008). *S. marianum* is an important medicinal crop in Europe, where it is mainly used to treat disorders and chronic diseases of the liver (Rambaldi et al., 2005; Freedman et al., 2011). In addition, it exhibited antioxidant properties both in vitro and in a rat animal model (Šeršeň et al., 2006; Nencini et al., 2007). The few studies on meat producing animals, showed that the inclusion of *Silybum marianum* in the diet of broiler chickens improved their immune response and enhanced their reproductive performances (Kalantar et al., 2014; Zarei et al., 2016). In addition, the breast and leg meat of supplemented chickens did not exhibit negative sensory attributes, thus having an overall sensory quality comparable to the meat of untreated animals (Šťastník et al., 2016). Despite such encouraging results, studies assessing the potential inclusion of milk thistle in the diet of meat producing animals are still limited. In particular, studies evaluating its potential application in the meat rabbit sector are absent.

Therefore, the aim of this trial was to study the effect of a dietary supplementation with a dried powder of *S. marianum* on the productive performance of growing rabbits, their health status and carcass traits. In addition, quality and sensory properties of the derived meat were evaluated.

2. Material and methods

The study was approved by the Institutional Animal Welfare Committee as the animal welfare body of the Kaposvár University. All animals were handled according to the principles stated in the EC Directive 86/60963/2010 EU regarding the protection of animals used for experimental and other scientific purposes (EC, 2010).

2.1. Animals and experimental diets

The study was carried out at the experimental farm of the Kaposvár University and a total of 144 Pannon Large rabbits (both sexes) of the Pannon Breeding Program were involved in the experiment. At weaning (35 days of age), animals were divided into three feeding groups: the control group (C, n=51 animals) was fed a basal diet (Table 1), whereas the other two groups received the control diet supplemented with two different concentrations of dried *Silybum marianum* (SM) which were 5 g/kg (SM1, n=48) and 10 g/kg (SM2, n=45). All diets had no anticoccidials or any other medications. Once formulated, the experimental diets were pelleted and stored at room temperature. The animals were housed in wire-mesh cages (3 rabbits/cage, size of cage: 61×32×30 cm, length×width×height). Temperature and photoperiod were 15–18 °C and 16L:8D, respectively. Animals were fed the experimental diets ad libitum from 5 to 11 weeks of age.

Body weights (BW) and average weight gain (AWG) were recorded based on the individual rabbit, whereas feed intake (FI) and feed conversion ratio (FCR) were calculated on the cage basis. Morbidity (diarrhoea, unkempt fur, bloody faeces and respiratory problems) and mortality were recorded daily. When calculating feed intake, it was assumed that morbid rabbits did not consume pellet for the two days before their death, hence they were not included in the feed intake calculations.

2.2. Slaughter and carcass dissection

At 11 weeks of age rabbits were transported to a slaughterhouse located 200 km far from the experimental farm. The duration of fasting was 6 h which included the transportation. Rabbits were electrically stunned and slaughtered by cutting the carotid arteries and jugular veins. Carcasses were then dissected according to the recommendations of the World Rabbit Science Association (WRSA), as described by Blasco and Ouhayoun (1996). The slaughtered rabbits were bled, and then the skin, genitals, urinary bladder, gastrointestinal tract, and the distal part of the legs were removed. Warm carcasses (with head, set of organs consisting of: thymus, trachea, oesophagus, lung, heart, liver, kidneys, and perirenal and scapular fat) were weighed and the ratio to slaughter weight (SW) was calculated. Carcasses were chilled at +4 °C and after 24 h were weighed (CC). The head and set of organs were removed from each carcass to obtain the reference carcass (RC). The RC included meat, bones, and fat deposits. Then the RC was cut between the 7th and 8th thoracic vertebra and between the 6th and 7th lumbar vertebra to obtain the fore, mid, and hind parts, which were weighed separately. The ratio of the head, organs, fat deposits and carcass parts to either CC or RC weights were calculated as required.

Hind legs (HL, right and left) and *Longissimus thoracis et lumborum* muscles (LTL, right and left) were dissected from 15 rabbits per dietary treatment (n=45 rabbits) and weighed. Then, they were individually vacuum-packed in polyethylene bags and kept at 4 ± 1 °C in portable refrigerators and transported to the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy) for meat quality analyses. Once in the laboratory, left LTL and HL were immediately frozen at −40 °C for further analysis.

2.3. HL and LTL pHu, colour, thawing and cooking losses

The right HL was deboned and the meat to bone ratio was calculated (Blasco and Ouhayoun, 1996).

Colour measurements (CIE, 1976) were carried out on the right LTL muscle (RM200QC colorimeter, X-Rite, Co., Neu-Isenburg, Germany) and considered lightness (L*), redness (a*) and yellowness (b*). Ultimate pH (pHu at 24 h *post mortem*) was measured in the right LTL meat and *Biceps femoris* muscle of the right HL, using a

Table 1
Chemical composition (g/kg as fed), mineral profile (mg/kg), vitamin content (mg/kg) and gross energy (MJ/kg) of *Silybum marianum* plant and of experimental diets.[1]

	Silybum marianum	Experimental diets[2]		
		C	SM1	SM2
Dry matter	939	912.2	904.3	910.4
Crude protein	159	169	170	165
Ether extract	241	25.4	26.4	25.9
Ash	43.4	74.7	77.6	75.7
Crude fibre	276	152	149	150
Neutral detergent fibre	380	324	326	316
Acid detergent fibre	292	203	202	196
Acid detergent lignin	99.5	55.6	57.3	51.7
Acid insoluble ash	0.70	11.2	12.7	10.2
Starch	4.70	172	168	180
Ca	6.57	6.96	7.63	6.65
P	4.91	5.93	5.90	5.70
K	4.21	10.1	10.6	9.87
Mg	2.76	3.47	3.93	3.36
Na	0.12	2.32	2.39	2.13
Fe	0.06	0.43	0.51	0.37
Zn	0.04	0.06	1.00	0.06
Ca/P	1.34	1.17	1.29	1.17
Vitamin E	117	39.0	51.0	37.0
Vitamin B1 (Thiamine)	0.86	0.87	0.87	0.93
Vitamin B2 (Riboflavin)	4.0	4.90	4.50	4.60
Gross Energy	23.6	18.0	18.0	17.9

[1] Analysed.
[2] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*, and SM2: C diet supplemented with 10 g/kg of *S. marianum*.

portable pH-meter (FG2-Five Go™ Mettler Toledo, Greifensee, Switzerland; calibration at pH 4.0 and 7.0). The pHu as well as the colour values represented the average of two repeated measurements. Right LTL were then vacuum-packed and stored at −40 °C until sensory analysis.

Frozen left HL were allowed to thaw overnight at +4 °C, and subsequently used for thawing and cooking loss determinations. After weighing, HL samples were individually vacuum-sealed using a CSV-41n ORVED machine (99% vacuum level) in polyethylene bags (water vapour transmission rate: 3.5 ± 1 g/m2 day at 23 °C and 85 ± 2% R.H.), and cooked in a water bath at 80 °C for 1 h. Afterwards, samples were cooled, dried and weighed.

2.4. Chemical analyses

The analyses of SM as well as those of the experimental diets (Table 1) were carried out in duplicate using the AOAC (2000) methods to determine the concentrations of dry matter (DM; Method no. 934.01), crude protein (CP; Method no. 2001.11), crude fibre (CF; Method no. 978.10), ash (Method no. 967.05) and starch (amyloglucosidase- α-amylase method, 996.11). Ether extract was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF, without sodium sulphite), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analysed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest et al. (1991), respectively, using the sequential procedure and the filter bag system (Ankom Technology, New York). The gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO, 1998). The mineral profile (Ca, P, K, Mg, Na, S, Fe, Zn) of the diets was analysed by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC, 2000, 999.10). The dietary content of vitamins E, B1 and B2 was analysed by EPTA NORD srl (via Padova, Conselve, Italy, internal methods n. PP 475 rev 4 2016, MI 234 rev 1 2014 and MI 235 rev 1 2014, respectively).

2.5. Measurement of lipid oxidation

After two months of storage, the left LTL (n=10 samples/treatment) were allowed to thaw for 24 h at +4 °C. They were then individually ground using a Retsch Grindomix GM 200 (7000 g for 10 s). The extent of muscle lipid oxidation was evaluated with a spectrophotometer (Hitachi U-2000, Theodor-Heuss-Anlage 12, Mannheim, F.R. Germany) set at 532 nm, that measured the absorbance of thiobarbituric acid-reactive substances (TBARS) and a 1,1,3,3-tetraethoxypropane calibration curve (Botsoglou et al., 1994). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

2.6. Sensory analysis

After 2 months of frozen storage, the 45 right LTL samples (15 per treatment) were subjected to a ranking sensory analysis, conducted by a four-member trained panel belonging to the MAPS Department. In order to familiarise with the food matrix and to select the appropriate descriptors, panel members underwent four pre-test training sessions, testing one rabbit loin/panellist/training session, purchased in a local supermarket. During the last two training sessions, panellists were also trained to familiarise with the ranking test and with the perception of dried ground *Silybum marianum* which was bought in a herbalist's shop. The test was carried out on three consecutive days: on each day of analysis, 15 samples were evaluated (5 samples×3 treatments) after thawing for 24 h at +4 °C. Vacuum-sealed samples (3 per PVC bag) were identified by a random three-digit code (C, SM1, SM2) and cooked in a water bath at 85 °C until core temperature reached 74 °C. Each cooked sample (still warm) was cut into four pieces of the same size and assigned to a panellist for the evaluation of sensory attributes. Each descriptor of the meat (rancid odour, herbaceous odour, rabbit odour, rancid flavour, herbaceous flavour and rabbit flavour) was ranked from the least (rank 1) to the most intense (rank 3).

2.7. Statistical analysis

Individual records of body weight, average weight gain and carcass traits were evaluated by one-way ANOVA of the statistical analysis software SAS, 2008, version 9.1.3) and processed choosing a mixed model that considered cage as random effect and treatment as fixed effect (PROC MIXED). FI and FCR data, calculated at cage level, were processed with a one-way ANOVA with the treatment as fixed effect (PROC GLM). Meat quality, TBARS and sensory analysis were processed with another one-way ANOVA with the treatment as fixed effect. A Chi-squared test with the Marascuilo (1966) procedure was performed on mortality data to detect the differences among the treatments. Bonferroni adjustments and three significance levels were assigned: *: $P < 0.05$; **: $P < 0.01$; ***; $P < 0.001$.

3. Results

The inclusion of *S. marianum* in the diets of growing rabbits did not affect their productive performance namely BW, AWG, FI and FCR (Table 2). Mortality rate was extremely high during the period 6–7 weeks, as a result of digestive problems, which accounted for the 69.1%, 49.8% and 71.6% of the whole mortality (5–11 weeks) for

Table 2
Effect of the supplementation of *Silybum marianum* on the live performance of growing rabbits.

	Experimental diets[1]			MSE[2]	Significance
	C	SM1	SM2		
Initial n rabbits/cages	51/17	48/16	45/15		
Age	Average body weight, g				
5 wk	883	886	894	16.6	ns
6 wk	1251	1267	1266	27.3	ns
7 wk	1493	1475	1462	53.2	ns
8 wk	1916	1876	1831	60.5	ns
9 wk	2335	2318	2287	60.3	ns
10 wk	2705	2720	2689	57.5	ns
11 wk	3067	3090	3067	62.3	ns
	Average weight gain g/d				
5–11 wk	52.3	52.5	51.5	1.35	ns
	Average individual feed intake, g/day/cage				
5–11 wk	145	145	142	19	ns
	Feed conversion ratio				
5–11 wk	2.88	2.86	2.85	0.17	ns
	Mortality, %				
5–6 wk	2.0	2.1	0	–	ns
6–7 wk	17.7[b]	10.4[a]	11.1[a]	–	*
7–8 wk	5.9	4.2	4.4	–	ns
8–9 wk	0	2.1	0	–	ns
9–10 wk	0	2.1	0	–	ns
10–11 wk	0	0	0	–	ns
5–11 wk	25.6[B]	20.9[B]	15.5[A]	–	**

Level of significance:
[1] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*; SM2: C diet supplemented with 10 g/kg of *S. marianum*.
[2] MSE= Mean Square Error.
* $P < 0.05$.
** $P < 0.01$ level.
[a, b, A, B] Means in the same row having different superscripts are significant at $P \leq 0.05$ and $P \leq 0.01$ levels, respectively; ns=non significant.

Table 3
Effect of Silybum marianum dietary supplementation on rabbit carcass traits.

	Experimental diets[1]			MSE[2]	Significance
	C	SM1	SM2		
n	30	32	34		
Slaughter weight (SW), g	2997	3058	2988	68.4	ns
Chilled carcass (CC), g	1810	1842	1785	46.1	ns
Reference carcass (RC), g	1517	1546	1490	40.8	ns
Chilled carcass yield, % SW	60.4	60.2	59.7	0.33	ns
Reference carcass yield, % CC	83.7	83.8	83.4	0.24	ns
Head, % CC	8.53	8.51	8.59	0.13	ns
HLTTO[(3)], % CC	1.38	1.35	1.37	0.04	ns
Liver, % CC	5.22	5.20	5.46	0.19	ns
Kidneys, % CC	1.02	0.98	1.06	0.03	ns
Perirenal fat% CC	0.93	0.95	1.06	0.06	ns
Scapular fat% CC	0.31	0.37	0.36	0.03	ns
Dissectible fat% CC	1.24	1.32	1.42	0.08	ns
Fore part, % RC	27.2	26.9	27.0	0.22	ns
Mid part, % RC	32.8	32.8	32.6	0.20	ns
Hind part, % RC	38.6	38.7	38.7	0.17	ns
Perirenal fat% RC	1.11	1.13	1.27	0.07	ns

ns=non significant.
[1] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*; SM2: C diet supplemented with 10 g/kg of *S. marianum*.
[2] MSE=Mean Square Error.
[3] HLTTO=Heart, lungs, thymus, trachea and oesophagus.

groups C, SM1 and SM2, respectively. In this challenging situation, mortality rate was significantly affected by SM inclusion: during the period 6–7 weeks, both SM1 and SM2 diets lowered the mortality rate of rabbits compared to the C group (17.7 vs. 10.4% and 11.1% for C, SM1 and SM2 groups, respectively; $P < 0.05$). Considering the whole experiment (5–11 weeks), global mortality was significantly lower in SM2 group (15.5%) compared to C and SM1 (25.6% and 20.9%, respectively).

The effect of *S. marianum* dietary supplementation on rabbit's carcass traits had not been studied before, and the results of the present experiment showed that its dietary inclusion at 5 and 10 g/kg levels did not affect the studied parameters (Table 3).

Also the rheological traits and pHu of the HL together with the colour and TBARS of the LTL muscle were not affected by the dietary inclusion of *S. marianum* (Table 4, 5). With increased SM supplementation, the pHu of the LTL muscle increased ($P < 0.05$). In fact, the meat of SM2 rabbits showed a higher pH compared to that of C group (6.10 vs. 5.98, respectively), with SM1 being intermediate.

The presence of *S. marianum* in the diet for growing rabbits affected the sensory characteristics of LTL meat (Table 6). Feeding animals with this plant increased the herbaceous odour ($P < 0.001$), whereas it lowered rabbit odour ($P < 0.05$) and flavour ($P < 0.001$). Specifically, herbaceous odour was higher in SM2 group compared to C and SM1 (2.18 vs. 1.90 and 1.93, respectively), rabbit odour was lower in SM1 and SM2 groups compared to C one (1.70 and 1.93 vs. 2.38, respectively) and rabbit flavour followed the same trend displayed by rabbit odour ($P < 0.001$).

Table 4
Effect of *Silybum marianum* dietary supplementation on the rheological traits of the hind leg (HL) meat of rabbits.

	Experimental diets[1]			MSE[2]	Significance
	C	SM1	SM2		
n	15	15	15		
HL weight, g	280	286	268	37.1	ns
pHu BF muscle[3]	6.07	6.12	6.19	0.19	ns
Meat to bone ratio	7.63	7.46	7.28	0.65	ns
Thawing loss, %	0.78	0.69	0.69	0.39	ns
Cooking loss, %	18.8	18.2	17.7	3.5	ns
Total losses, %	19.5	18.9	18.4	3.6	ns

ns= non significant
[1] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*; SM2: C diet supplemented with 10 g/kg of *S.marianum*.
[2] MSE= Mean Square Error.
[3] pH measured 24 h *post mortem* on the *Biceps femoris* muscles.

Table 5
Effect of *Silybum marianum* dietary supplementation on pHu, L*a*b* colour values, and TBARS contents (mg MDA/kg muscle) *of Longissimus thoracis et lumborum* (LTL) meat of rabbits.

	Experimental diets[1]			MSE[2]	Significance
	C	SM1	SM2		
n	10	10	10		
TBARS	0.23	0.21	0.21	0.03	ns
pHu LTL muscle[3]	5.98[a]	6.03[a,b]	6.10[b]	0.12	*
L*[3]	51.4	51.1	48.9	3.04	ns
a*[3]	3.37	2.80	2.41	1.20	ns
b*[3]	0.12	0.06	−0.28	0.88	ns

Level of significance:
[1] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*; SM2: C diet supplemented with 10 g/kg of *S.marianum*.
[2] MSE= Mean Square Error.
[3] Measured 24 h *post mortem* on LTL muscles of all the slaughtered rabbits.
* $P < 0.05$.
[a, b] Means in the same row having different superscripts are significant at $P \leq 0.05$; ns=non significant.

Table 6
Effect of Silybum marianum dietary supplementation on the sensory analysis of Longissimus thoracis et lumborum (LTL) meat of rabbits.

	Experimental diets[1]			MSE[2]	Significance
	C	SM1	SM2		
n	15	15	15		
Rancid odour	2.08	1.98	1.95	0.82	ns
Herbaceous odour	1.93[A]	1.90[A]	2.18[B]	0.72	***
Rabbit odour	2.38[b]	1.93[a]	1.70[a]	0.78	*
Rancid flavour	2.28	1.83	1.90	0.82	ns
Herbaceous flavour	2.03	1.88	2.10	0.82	ns
Rabbit flavour	2.40[B]	1.90[A]	1.70[A]	0.73	***

Level of significance:
[1] C: control diet; SM1: C diet supplemented with 5 g/kg of *S. marianum*; SM2: C diet supplemented with 10 g/kg of *S. marianum*.
[2] MSE= Mean Square Error.
* $P < 0.05$.
*** $P < 0.001$ level.
[a, b, A, B] Means in the same row having different superscripts differ at $P \leq 0.05$ or $P \leq 0.001$, respectively; ns=non significant.

4. Discussion

Independently of the dietary treatment, the productive performance of growing rabbits were satisfactory overall and in line with results presented in other studies considering Pannon Large rabbits (Szendrő et al., 2016). The body weight of rabbits at 5 weeks of age was lower in our study compared to data published by the same authors, but rabbits of the present experiment had a higher average weight gain, thus reaching higher body weight at 11 weeks of age. Studies on broiler chickens showed that the effect of *S. marianum* dietary supplementation on the productive performance is controversial because results differed (Schiavone et al., 2007; Mojahedtalab et al., 2013; Kralik et al., 2015b; Kalantar et al., 2014; Morovat et al., 2016), as a result of differences among these studies regarding the extraction method, the active components and the treatment manner.

The post-weaning period, from five to eight weeks of age, is known to be critical for the rabbit due to the impact on the developing

digestive system (caecal microbiota) and consequently on the mortality rate that may compromise the fattening period (De Blas et al., 2012). After the ban of the antimicrobial growth promoters (AGPs), several researchers started to test extracts from plants and spices as supplements in the diet for growing rabbits, trying to guarantee satisfactory performance (Dalle Zotte et al., 2013, 2016; Cardinali et al., 2015; Celia et al., 2016a). Indeed, herbal drugs have a wide range of health-related properties, among them, those related to disease prevention. *S. marianum* has been traditionally used as a natural remedy in liver and gastrointestinal diseases (Saller et al., 2002). However, up to now there were no experiments or evidence demonstrating the efficacy of *S. marianum* in improving the intestinal health of rabbits, and the results of the present research suggested that it might improve health status of rabbits with challenging digestive pathologies. Studies by Vogel et al. (1984) and Kalantar et al. (2014) found a reduction in the mortality rate of dogs intoxicated with *Amanita phalloides* and treated with an intravenous injection of 50 mg/kg silibinin, and a reduction of ileum pathogenic bacteria in treated chickens (0.5% dietary inclusion), respectively. Regarding in vitro studies, Abed et al. (2015) observed a significant effect of a seed extract from *Silybum marianum* against pathogenic bacteria such as *Staphylococcus saprophyticus, Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae*.

As in another experiment with Pannon Large rabbits (Szendrő et al., 2016), the hind part had a higher impact on the RC compared to fore and mid parts, and the slaughter weight at 12 weeks of age was comparable to that of Pannon Large rabbits reported by Dalle Zotte et al. (2015) experiment. Other authors investigated the effect of a dietary supplementation with different extracts from *S. marianum*, but only in broiler chickens; generally the dietary inclusion did not affect carcass traits (Colturato et al., 2012; Kralik et al., 2015b), but Schiavone et al. (2007) observed a negative effect on feed intake and carcass weight of birds, when a dried extract of *S. marianum* fruits was supplemented at 40 and 80 ppm in the diets.

Thawing and cooking losses of SM1 and SM2 HL meat were comparable to those of conventionally fed rabbits which was important from the technological point of view. *Biceps femoris* is considered one of the least oxidative intermediary muscles, hence its high pH (Hulot and Ouhayoun, 1999). Other authors have also found that diets supplemented with plants rich in flavonoids did not have relevant effects on rheological traits of rabbit meat (Dalle Zotte et al., 2014; Simitzis et al., 2014). Growth parameters and some carcass characteristics (i.e. intramuscular fat content) are correlated to some rheological traits such as pH, WHC and thus water losses (Hernández et al., 2000; Dalle Zotte, 2002; Celia et al., 2016b). Consequently, as carcass attributes were not statistically different among dietary groups of the present experiment, it was expected that the rheological traits followed the same trend.

It was interesting to notice that with increasing SM inclusion in the diet, the pHu of LTL muscle tended to increase and became higher in SM2 compared to C meat. Silibinin, the active component of *S. marianum*, was reported to affect energy metabolism of treated male Wistar rats, in a dose-dependent manner; it inhibited gluconeogenesis in fasting condition and glycolysis in fed condition (Colturato et al., 2012). LTL muscle has prevalent glycolytic metabolism, hence the observed pHu increase in LTL of SM fed rabbits might be dependent on the above mentioned metabolic pathway. A higher pHu was also observed in breasts meat of broiler chickens fed with 3% milk thistle oil compared to those receiving 3% sunflower oil (Kralik et al., 2015a) and, also in this case, a change in glycolytic processes was hypothesized.

S. marianum supplementation did not show antioxidant effect, which was somewhat surprising. In fact, its antioxidant activity was proven in broiler chickens fed with 40 and 80 ppm of a dried extract of the plant fruit, as well as in rats fed for 3 days with 200 mg/kg diet of silymarin, where a protective effect on antioxidant defence systems was observed (Schiavone et al., 2007; Nencini et al., 2007). Silymarin demonstrated its antioxidant effect also in an in vitro study where different antioxidant assays were evaluated (Köksal et al., 2009). Flavonoids are considered a good source of natural antioxidants that positively affect meat quality (Dal Bosco et al., 2014). However, the antioxidant capacity and bioactive compounds as well as in vivo efficacy is determined by many factors such as the part of the plant which is used (eg. stems, leaves or heads), the growing stage of the plant, the dose-dependent effect (Sulas et al., 2016; Saeed et al., 2012), thus possibly explaining our findings.

From the sensory point of view, flavonoids influence human food preference as they are important olfactory agonists thus affecting also taste sensation, which is parallel to the olfactory one. In fact, everyday sensory perceptions such as the aroma of freshly brewed coffee, the bouquet of a wine, etc., are mainly due to flavonoids (Havesteen, 2002). Hence, feeding animals with a plant rich in flavonoids may affect the sensory characteristics of the derived meat, which was observed in the present study for herbaceous and rabbit odours, and rabbit flavour. Their presence might be so peculiar that panellists differentiate meat of animals fed with or without flavonoid-rich diets; this was the case of the study by Beghelli et al. (2014), in which the meat of pigs supplemented with oregano was always recognized different from a control group. Similarly, Nieto et al. (2011) found that the inclusion of thyme leaves in the diet of pregnant sheep positively affected the sensory characteristics of cooked lamb meat. In the experiment of Šťastník et al. (2016), panellists declared a finest quality for colour and fibrous parameters of the thigh meat of broiler chickens fed with 5% or 15% milk thistle seed cakes. However, literature data showed also that the direct inclusion of natural compounds in the meat or in the diet of animals is not always perceptible (Bianchi et al., 2009) or it can also be associated to unfavourable characteristics (Cullere et al., 2015; Celia et al., 2016b).

5. Conclusions

Silybum marianum dietary supplementation reduced the mortality rate in growing rabbits under health stress, thus being a promising natural feed additive in improving the sanitary status of a commercial rabbit farm. The dietary supplementation with *Silybum marianum* changed the sensory characteristics of rabbit loin thus, in the future, consumer acceptability should be carefully assessed. As the present study was the first attempt to test the dietary supplementation of *Silybum marianum* in the diet for growing rabbits, further studies need to implement the present results considering also digestibility of nutrients as well as the effect of this herb on the intestinal microbiota.

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Conflict of interest

Authors declare that there's no financial/personal interest or belief that could affect the objectivity of the present research study. Therefore, authors declare that no conflict of interest exists.

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8. GENERAL DISCUSSION

Antibiotic were used for decades in the rabbit meat production, to diseases prevention and production enhancement. However, discovering of a theoretical connection on the development of resistant bacterial strains, have revealed doubts on their utilization. In 2006 they were finally banned from the European countries, opening the Era of the natural products.

Studies on the digestive disturbances of growing rabbit have revealed how nutrition plays an active role in maintaining a positive health status. Indeed, microbial colonisation of the rabbit gastrointestinal tract is directly related to a supply of balanced diets, and any alteration may provoke the colonisation of pathogenic bacteria, primary cause of digestive disturbances.

Different strategies were explored to reduce the use of the antibiotics, through feed restriction, modern management techniques, and natural feed supplements. Among the last category, the candidate might be probiotics, prebiotics, organic acids and, in particular, plants and their extracts.

Plants have played a significant role in maintaining human health and improving the quality of human life for thousand of years. It was estimated that more than 80% of the Earth population rely in traditional medicine for their primary health care need, and mostly the use of plant extract is involved. Thus, the aim of the experiments included in this PhD thesis was to find positive effects of some herbs and spices supplemented to rabbits diets, in particular on the health status, growth potential and meat quality of the growing rabbits.

The first study showed the single and/or synergistic effect of the phytochemicals included in the Digestarom®, a mixture of essential oils, herbs, spices and extracts of 10 different ingredients: onion (*Allium cepa* L.), garlic (*Allium sativum* L.), caraway (*Carum carvi* L.), fennel (*Foenicum vulgarae* L.), gentian (*Gentiana lutea* L.), melissa (*Melissa officinalis* L.), mint (*Mentha arvensis* L.), anise (*Pimpinella anisum* L.), oak bark (*Quercus cortex*) and clove (*Syzygium aromaticum* L.). Each ingredient contains different phytochemicals, mainly phenolics and flavonoids substances which produces a different effect due to different mechanisms. It was observed how phenolic substances present in the mix had influenced positively the live performances in the post-weaning period, improving feed efficiency and growth rate. On the other hand, tannins-like substances naturally present in oak bark had negatively influenced the palatability of the diet, and impairing the nutrient digestibility.

The most important mechanism of phytogenic feed additives is claimed to be the beneficially effect on the ecosystem of gut microflora through controlling potential pathogens. Digestarom® supplementation lowered the mortality trend after weaning, but the the microbial count analysis did not reveal positive change in the microbiota, differently from the results of the literature. The presence of phytochemicals in the Digestarom® had nearly no effect on carcass and meat quality traits in measured in the second study. Only flavour and taste perception was likely affected by the presence of the aromatic ingredients such as allicin, component of onion and garlic. The pungent aroma of allicin was not appreciated because the panelists associated it with olfactory and flavour rancidity.

In the third study, the supplementation of *Silybum marianum* (milk thistle) in the diet of growing rabbit was able to significantly reduce the

mortality, mainly in the delicate post-weaning phase. Traditionally, milk thistle is used for protecting and restoring liver function, because of the high content of flavonoids are claimed to promote antioxydative and anti-inflammatory actions, and to help in reducing the risk of diseases. Surprisingly, antioxidative action was not detected in the meat of the rabbits fed with *Silybum marianum*, as occurred in other animal species. Possible factors, such as animal species, age, type of plant extract, and inclusion level, might have interfered with the effect of the phytochemicals, making useless its supplementation to this purpose.. Differently, flavonoids affected positively some meat sensory traits, permitting the panelists to differentiate the meat of rabbits fed with or without flavonoids-rich diet. Therefore, *Silybum marianum* might be considered a potential feed supplement for growing rabbits, considering its ability of lowering the mortality of the rabbits around weaning.

In all the studies included in this PhD thesis both positive and absence of effects the phytochemicals were found. To formulate diets using natural ingredients, it is preventively important to evaluate possibly side effects, as astringency, toxicity and tolerance level, however not always easy to determine. Indeed, when phytobiotic additives are added as feed supplements, different parameters can occour to modify the helpfulness: plant parts and physical properties, genetic variety of the plant, the level of dosage, harvest time and interaction with the other ingredients. In addition, the efficacy of the phytobiotic additives might be affected by the nutritional status of the animals, infections and diet composition.

It can be concluded that the future of using herbs and/or spices in rabbit feeding will, in great measure, depend on the knowledge of their chemical structure, economical value, and technological advancements for their use in pelleted diets.

9. CONCLUSION

Several herbs, spices, and botanicals products have been tested, as feed supplement, in the growing rabbits with disparate results. Some of them have shown beneficial effects in rabbit live performances as growth promoter, others exhibited antimicrobial and antioxidant properties, whereas others improved the meat sensory traits.

The administration of 300 mg/kg of Digestarom® in a diet for growing rabbits proved to be mainly effective after weaning (from 5 to 8 weeks of age), as it reduced the mortality rate, and improved feed efficiency and growth rate. However, it impaired nutrient digestibility and some meat sensory traits. Also the dietary supplementation of *Silybum marianum* to growing rabbits had, as main effect, the reduction of mortality after weaning.

In conclusion, results of the present PhD thesis have demonstrated a weak effectiveness of the use of both supplements as natural feed additive for growing rabbits, and their use would be suggested around weaning, to improve the health status of commercial rabbits.

10. NEW SCIENTIFIC RESULTS

1. The dietary supplementation of 300 mg/kg of Digestarom® significantly reduced the DM intake. As the tannin content of Digestarom® is supposed to be responsible for that effect, it is suggested to exclude the oak bark in the commercial mix.

2. The dietary supplementation of *Silybum marianum* herbal powder at 5 and 10 g/kg inclusion level reduced the mortality rate of rabbits during post-weaning , thus being a useful natural feed additive in improving the sanitary status in commercial rabbit farms.

3. The use of 5 and 10 g/kg *Silybum marianum* in rabbit diets significantly increased the herbaceous odour ($P<0.001$), whereas it lowered the rabbit odour ($P<0.05$), and flavour ($P<0.001$). However, to evaluate the sensory traits of this herb, consumer acceptability should be carefully assessed.

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13. PUBLICATIONS & PRESENTATIONS SCIENTIFIC PAPERS ON THE SUBJECT OF THE DISSERTATION

Peer-reviewed papers published in foreign scientific journals

Celia C., Cullere M., Gerencsér Zs., Matics Zs., Dalle Zotte A., Giaccone V., Szendrő Zs., 2015. Effect of Digestarom® dietary supplementation on the reproductive performances of rabbit does: preliminary results. Ital. J. Anim. Sci. 14, 700-705.

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Poster

Celia C., Szendrő Zs., Matics Zs., Gerencsér Zs., Cullere M., Tasoniero G., Dalle Zotte A., 2015 effect of Digestarom® feed additive on rabbit carcass traits and meat sensory attributes. Proceedings of the 61st International Congress of Meat Science and Technonlogy, August 23-28th 2015, Clermont-Ferrand, France 6.30.

OTHER PUBLICATIONS NOT RELATED TO THE TOPIC OF THE DISSERTATION

Peer-reviewed paper published in foreign scientific journal

Kachlek M., Szabó-Fodor J., Szabó A., Bors I., Celia C., Gerencsér Zs., Matics Zs., Szendrő Zs., Tuboly T., Balogh-Zándoki E., Glávits R., Dalle Zotte A., Kovács M., 2017. Subchronic exposure to deoxynivalenol exerts slight effect on the immune system and liver morphology of growing rabbits. Acta Vet. Brno 86, 37-44.

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Kachlek M., Szabó-Fodor J., Bonai A., Bors I., Celia C., Gerencsér Zs., Matics Zs., Szendrő Zs., Dalle Zotte A., Kovács M., 2015. Accessing the possible interaction between card*uus marianus* and dietary deoxynivalenol on caecal microbiota and fermentation of growing rabbits. Proceedings of the 23rd international symposium "Animal science days" 21-24th September, 2015, Istra-Neptun – National Park BRIJUNI, Croatia pp. 186-189.

Poster

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Effectiveness of different plant sources in improving the shelf-life of chicken meat patties. Proceedings of the 61st International Congress of Meat Science and Technonlogy, 23-28th August 2015, Clermont-Ferrand, France 7.53.

14. CURRICULUM VITAE

Chiara-Carmen Celia was born in Montebelluna, (TV) Italy on 5th of January 1987.

In July 2007 she obtained the High diploma specialising in scientific subjects in the Scientific high school "Liceo Primo Levi".

In October 2007 she started the Bachelor in Animal Science and Technology in Padova University.

Between March 2010 and September 2010 she was a veterinary helper in dairy cattle farms with the supervision of prof. Massimo Morgante.

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Between April-June 2011 she worked as Livestock controller in the company "Colomberotto Carni".

In October 2011 she started the Master in Animal Science and Technology in Padova University.

In July 2013 she obtained the Master Degree in Animal Science and Technology in Padova University with the thesis: "Application of near infrared spectroscopy to assess fillets quality of rainbow trout (*Oncorhyncus mykiss*)".

Between August 2013 and August 2016 she he was a full-time student at the Doctoral School of Animal Science of Kaposvár University.

Between June 2014 and September 2014 she participated to the Marie Curie Scholarship "Herbal protection".

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Bewteen November 2015 and May 2016 she obtained an Erasmus+exchange programme grant to perform meat analysis experiments in Padova University.

In May 2016 she completed her comprehensive exam to obtain the pre-doctoral status (fulfilled *summa cum laude*).

In January 2018 she started to work in the Genetics department of the Institute for Diabetes and Obesity in the Helmholtz centrum, Munich.

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