UNIVERSITÀ DEGLI STUDI FIRENZE

DOTTORATO DI RICERCA IN
Scienze agrarie e ambientali

CICLO XXXI

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Cinta Senese pig breed: protein requirements in post-weaning and characterization of two processed pork products enhanced through the addition of omega-3 and natural antioxidants

Settore Scientifico Disciplinare AGR/19

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Anni 2015/2018

Abstract

The thesis was included in a larger project ("Diversity of local pig breeds and production systems for high quality traditional products and sustainable pork chains", European Union's Horizon 2020 research and innovation program under grant agreement No 634476, acronym TREASURE). The overall aim of TREASURE was to enhance the knowledge of local pork chains, starting from the rearing systems, feeding strategies, nutritional requirements and meat quality traits. The project considered also the pork products in a future perspective, proposing innovation to the traditional recipes in order to address consumers' demand for healthier, eco-friendly and animal-friendly products. The present thesis approached two main issues of Cinta Senese pork chain, the scarce knowledge on nutritional requirements during the growing phase and the changing demand for healthier pork products.

Cinta Senese, as most of the local pig breeds, belongs to the obese pig genotype that have specific metabolic features and, consequently, specific nutritional requirements, markedly different from cosmopolite breeds' ones. However, Cinta Senese is bred by a small number of farmers and few studies, especially on its nutritional requirements, were carried out. The lack of research leads to unbalanced feeding formulations along the whole life cycle. The first part of the present work was aimed to identify the optimal protein supply for Cinta Senese growing pigs, also considering that during the growing and early fattening stages, pasture supplementation with concentrate is widely applied. Identifying the optimal protein requirements of Cinta Senese growing pigs is of central importance to avoid economic losses, as well as to lower the environmental impact of this rearing system. Maintaining unchanged the quality of protein provided, four protein levels were tested: 12, 14, 16 and 18% of crude protein. The first trial (Trial 1) presented in this thesis concerned the evaluation of the experimental diets in terms of *in vivo* performances, slaughtering traits and nitrogen balance. The results obtained have indicated the 12% CP diet as adequate to fulfil protein requirements during the growing phase. These results corroborated earlier studies on Cinta Senese regarding different growth stages, but they also agree with most of the studies on other European local pig breeds, that showed similar protein requirements.

The second trial (Trial 2) dealt with the improvement of Cinta Senese meat and meat products. These are considered niche products, being spread almost exclusively within

the territory in which this breed is traditionally reared and, due to the peculiarities of its rearing systems, they are also averagely expansive. Moreover, fresh meat market is currently poorly developed. Indeed, Cinta Senese, as all obese genotypes, is characterized by a great predisposition to deposit fat instead of lean tissues, leading to a carcass noticeably fat. These factors contribute to diminish the appeal of fresh meat, that is considered unhealthy due to its fatness. This second part of the work investigated the feasibility of introducing some innovative ingredients in two Cinta Senese pork products, the burger and a type of dry-fermented sausage. Burgers were chosen being considered a wide-appreciated and easy-to-cook fresh product. The novelty proposed was aimed to enhance the fatty acid profile by the addition of EPA and DHA, two essential omega-3 polyunsaturated fatty acids. The enrichment was carried out testing two different techniques: i) adding directly the deodorized fish oil or ii) adding the fish oil previously microencapsulated by spry-drying. The latter technique resulted the most appropriate to preserve the added omega-3 from lipid oxidation during storage and cooking, as well as to avoid extraneous flavors in the final product. Addressing the increasing concerns on the employment of nitrite and nitrate as curing agent in processed products, two different mixture of natural antioxidants were tested in the dry-fermented sausages as sodium nitrite replacement. The vegetal extracts were obtained by olive pomace, grape seed and chestnut. The physical, chemical and sensorial characteristics of the novel products were studied, as well as their aromatic profile. The results obtained suggested that sodium nitrite replacement is practicable, indeed, no differences in microbiological safety, lipid oxidation and aromatic profile were observed. Though, the replacement had some negative effects on instrumental color, being nitrite pivotal in developing the typical redness of processed pork products.

In conclusion, Cinta Senese rearing system, as many of Mediterranean silvo-pastoral systems, already presents a plenty of characteristics that perfectly matche with the novel societal demand for animal welfare, return to traditional rearing techniques and genuine products. In this perspective, the local pork chains still have a great untapped potential. Increasing the knowledge about all their parts (rearing, feeding and processing), would help stakeholders to make the whole production chain also economic affordable and environmental respectful.

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GENERAL INTRODUCTION

1. Local pig breeds in Europe and in Italy

After the end of World War II, agriculture industrialization and intensification processes deeply changed the farming systems and market demand. The increased demand for animal products and the introduction of innovative management and nutrition strategies, led to the decline of native breeds that were replaced by selected breeds, well-adapted to the new farming systems and able to guarantee high performances in lesser time. Several local breeds started declining and most of them got extinct. This took place for all the livestock species and, among, them also pigs were involved. Hundreds of native pig breeds disappeared, this caused also a loss of genetic resources all over Europe. In the last decades a new attention to biodiversity has risen and the conservation of animal germplasms, including livestock ones, became pivotal, as outlined during the 1992 Convention of Rio de Janeiro.

Local pig breeds were not able to compete with selected breeds, such as Large White, Landrace and Duroc, in terms of performances, growth rate and slaughter yield, but their rusticity and the intrinsic quality traits of their meats, can still be considered an untapped potential to be exploit.

According to FAO database, most of the European countries have local pig breeds, but in several cases, data on the population sizes and reliable information are not available. Table 1 shows size and risk level (FAO, 2018) of that European native pig breed populations with reliable data. Besides the Iberian pig (Figure 1) in Spain (that cluster a group of native breeds genetically very close), the Alentejano and Bisaro in Portugal and the Blond Mangalica in Hungary, most of the other breeds are currently consisting of small populations. This means that a great part of them requires collective management both for maintenance of the breed and to avoid inbreeding (Pugliese and Sirtori, 2012). Even though, the portraited situation is valid for the most of European native breeds, a positive exception can be found for the Iberian pig

Figure 1. Iberian pig

breed. In this case, the strong cooperation between public institutions, producers and researchers, have maintained the population of this native pig far above the risk of extinction or inbreeding, also valorizing the Iberian pork products, which are well known throughout Europe and beyond.

Table 1. Size and risk level of European native pig breeds (FAO, accessed on September 4th, 2018)

	Number min/max				Risk
	1997	**2009**	**2012**	**2016**	
Spain					
Chato Murciano	20/30	100/1000	162/162	3251/3251	Yes
Euskal Txerria	-/100	-	45/45	171/171	Yes
Gochu Asturcelta	-	982/982	1551/1551	1530/1530	Yes
Ibérico	1000/10000	133175/133175	29252/29252	560591/560591	No
Negra Canaria	100/1000	609/609	561/561	533/533	Yes
Porc Negre Mallorquì	700/700	1217/1217	1116/1116	1077/1077	Yes
Porco Celta	8/100	4934/4934	4591/4591	2397/2397	Yes
Portugal					
Alentejana	4350/-	9000/13000	25093/31398	32680/40913	No
Bisaro	182/-	2200/3000	24714/31441	32237/41003	No
Malhado de Alcobaca	-	80/80	438/557	1170/1489	Yes
France					
Blanc de l'Ouest	100/1000	-	-	58/58	Yes
Creole de Guadeloupe		-	-	129/129	Yes
Cul Noir limousin	10/1000	-	-	95/95	Yes
Gasconne	100/1000	-	-	926/926	Yes

Table 1. Size and risk level of European native pig breeds (FAO, accessed on September 4th, 2018)

	Number min/max				Risk
	1997	**2009**	**2012**	**2016**	
Nustrale	2500	-	-	372/372	
Porc de Bayeux	63/100	-	-	60/60	Yes
Germany					
Bunte Bentheimer	82/82	314/314	320/320	587/587	Yes
Sattelschweine		90/90	757/757	963/963	Yes
Rotbuntes Husumer Schwein	110/-	74/74	31/31	-	Yes
Schwäbisch Hällisches	207 / 207	-	-	-	Yes
United Kingdom					
Berkshire	277/-	722/722	680/680	407/407	Yes
British Lop	252/-	352/352	450/450	435/435	Yes
British Saddleback	601	922/922	882/882	504/504	Yes
Gloucestershire Old Spots	522	1277/1277	1627/1627	708/708	Yes
Large Black	334	363/363	362/362	333/333	Yes
Middle White	257	416/416	391/391	345/345	Yes
Oxford Sandy and Black	180	387/387	684/684	543/543	Yes
Tamworth	354	499/499	502/502	384/384	Yes
Welsh	470	444/444	793/793	692/692	Yes
Slovenia					
Krskopolje	136	658/658	821/821	1950/1950	Yes
Latvia					

Table 1. Size and risk level of European native pig breeds (FAO, accessed on September 4th, 2018)

	Number min/max				Risk
	1997	**2009**	**2012**	**2016**	
Latvijas Baltā	27500/360000	-	-	100/200	Yes
Netherlands					
Bentheimer varken	-	-	100/100	420/420	Yes
Hungary					
Blond Mangalitsa /	961/961	4376/4376	4463/4463	-	No
Red Mangalitsa	136/136	1303/1303	1949/1949	-	Yes
Swallow Bellied Mangalitsa	131/131	785/785	1166/1166	-	Yes
Greece					
Greek Black pig	-	-	-	-	
Croatia					
Banijska šara	-	-	-	50/100	Yes
Black Slavonian pig	100	1200/1300	1200/1300	1800/1900	Yes
Turopolje pig	38	200/250	200/250	150/200	Yes
Serbia					
Mangulica	55/100	1000/2000	100/1000	2000/4000	Yes
Moravka	500/1000	100/500	150/500	500/1000	Yes
Resavka	50/1000	50/100	50/100	100/200	Yes

According to Lopez-Bote (1998), from the 1950s to 1970, a number of factors linked to urban development and the intensification of animal production reduced the consistency of Iberian pigs. In recent years, the demand for Iberian pig products has increased, which is attributed to a revaluation of traditional, top-quality products. This trend is shared by most of the others native breeds, whose survival is still not certain and strongly depends

on the possibility to valorize their production systems to obtain high-added value products, widely known and appreciated by consumers, following the Iberian's footsteps.

Besides the Iberian pig, Spain accounts for other native breeds such as the Chato Murciano, the Esukal Txerria, el Negra Canaria and the Porc Negre Majorquì, which have been or currently are involved in recovery programs promoted by policy makers and they are also characterized by an increasing market's demand (Franco *et al.*, 2006; Poto *et al.*, 2007; Gonzalez *et al.*, 2013; Olalde *et al.*, 2015).

Figure 4. Alentejano pig

Figure 3. Bisaro pig http://www.porcobisaro.net)

In Portugal, according to the FAO database, three native pig breeds are currently under census: Alentejano (Figure 2), Bisaro (Figure 3) and Malhado de Alcobaca, but only the last one is considered at risk of extinction. The Alentejano and Bisaro breeds, thanks to the high-quality products and consumers' appreciation are widely bred in the country; moreover, Alentejano have also a PDO on fresh meat (Teixeira and Rodrigues, 2013; Martins *et al.*, 2015). In recent years, Portugal traditional rearing systems are changing and a growing interest in native pig breed are developing, determining an expansion of native pig breeds with many changes in production, processing and marketing (Matos, 2000). In France, 7 native pig breeds were identified. Thanks to the jointed action of stakeholders, all breeds are under conservation actions, but as for the most of these breeds, the population size and the risk of inbreeding, make conservation difficult (Lauvie *et al.*, 2011).

Figure 2. Noir de Bigorre pig

Among them, Gasconne, Noir de Bigorre (Figure 4) and Nustrale have a PDO on meat or/and ham.

In Greece, only one native pig breed is recognized, the Greek black pig, providing fresh meat and cured products of excellent quality and great organoleptic characteristics. Currently, few breeders are raising this breed intensively, with their farms counting almost 300 sows in total, which place the Greek black pig on the list of endangered autochthonous breeds (Michailidou *et al.*, 2014).

The only native pig breed registered in Slovenia is the Krškopolje (Figure 5), or "blackbelted", originated in Dolenjska, in the south-eastern portion of the country. The interest in this breed increased in the last 10 years and now it is included in the national preservation program for autochthonous breeds (Čandek-Potokar *et al.*, 2003).

Figure 5. Krškopolje pig (https://treasure.kis.si/krskopoljski-krskopoljska/)

Croatia counts two main autochthonous breeds, the Black Savonian and the Turopolje (Figure 6), FAO database reports also a third breed, the Banijska šara, whose first census took place in 2016 and it was recently recognized as a Croatian native breed. The Black Slavonian was the most widespread breed in Slavonia, a region in eastern Croatia and it was used mainly for fat and meat productions. Due to the current protection measures, the effective population increased; in 2017, there were 242 boars and 1930 sows (Croatian agricultural agency, www.hpa.hr, accessed on November 30th, 2018).

Figure 6. Turopolje pig

Figure 7. Schwäbisch-Hällisches pig

In Germany, the oldest domestic pig breed is originated from Schwäbisch Hall in Baden-Württemberg. The Schwäbisch-Hällisches pig breed (Figure 7) likely developed around 1821 as a crossbreed between Chinese pigs and local ones. After a period of decline due to the introduction of English selected breeds, in 1920s the Schwäbisch-Hällisches breed association was established and the breed started again to increase its number. However, as almost all the European native breeds, after the World War II, the association closed and in 1980s the breed was nearly extinct. In recent years a new association was funded and the herdbook was re-opened accounting only few sows and boars. During the following years, thanks to the activity of the association and the regional marketing program, aimed to sell Schwäbisch-Hällisches's meat as premium quality product, the breed numbers increased again, accounting now 350 breeding sows and 35 boars. (Personal communication). UK has a long tradition in pig breeding, several native breeds are still present in the country either for production than for affection. The most widespread local breeds in terms of numbers of animals is the Gloucestershire Old Spots (Figure 8) originated in the Berkeley Vale of Gloucestershire, where it was known as the Orchard Pig, it used to graze in the apple orchards, clearing up the windfalls as well. Due to its rusticity and long tradition of grazing, it is well suited to outdoor rearing systems. In the last decades, the breed was involved in a program aimed to record and test the reproducers. Its meat is considered of high quality and its demand is increasing, so that many butchers are now specializing in it. From being a very small breed 40 years ago, it is now the largest numerically of the pig breeds listed by The Rare Breeds Survival Trust, the breed currently has 4 male and 15 female lines (http://www.britishpigs.org.uk/, accessed on September 4th, 2018).

Figure 8. Gloucestershire Old Spots pig

Figure 9. Blond Mangalitsa pig (https://www.mangalitzainternational.org)

The Mangalica breed was the most important breed in Hungary, with 30000 breeding sows in 1943 and about 18000 in 1955 (Egerszegi *et al.,* 2003). The consistence of this breed dramatically dropped until the 1990s, when only 154 - 346 registered sows remained. In 1996, a Hungarian state program was established to propagate the Mangalica breed. Today, about 8000 Mangalica breeding sows are kept again (Ratky *et al.*, 2013).

Nowdays, there are three indigenous pig breeds in Serbia: Mangalitsa, Moravka and Resavka. They are mainly bred in small pig farms (up to ten sows) that cannot compete with the intensive ones (with a hundred and more sows). Small breeders are increasingly starting to grow native breeds in outdoor systems (organic pig farming, semi-intensive or intensive systems) to produce meat and local products such as "Srem kulen" and "Sremska sausage" (Savić *et al.*, 2018).

In Italy, at the beginning of the XX century, there were numerous local pig breeds, but only six survived to the changes in production systems and the introduction of selected breeds. They are: Apulo-Calabrese, Casertana, Cinta Senese, Mora Romagnola (Figure 10), Nero Siciliano and Sarda, and, currently, they are the only ones to have a Pedigree Register, and, consequently, can be considered as effective breeds (Table 2). In the last decade, the growing demand observed for meat and meat products derived from these breeds is a result of the increased interest of Italian consumers for "niche products" (Pugliese and Sirtori, 2012). Alike the other breeds reared in the Mediterranean basin, which share their origins with the Italian ones, they are all characterized by colored coat and a high tendency for fat infiltration into muscle (Ollivier *et al.*, 2001).

The population sizes of the Italian local breeds over the last decade, show a moderate increase for the smallest populations and a slightly decrease for the biggest ones (i.e. Cinta Senese). This is likely due to the reaching of an equilibrium in the niche product

markets. All the breeds are mainly reared in their original areas, but Cinta Senese is now starting to spread also out of Tuscany (Franci and Pugliese, 2007).

Table 2. Size and risk level of Italian native pig breeds (FAO, accessed on September 4th, 2018)

	Number min/max				Risk
	1997	**2009**	**2012**	**2016**	
Casertana	30	366/366	386/386	604/604	Yes
Cinta Senese	100/500	2152/2152	2502/2502	2613/2613	Yes
Macchiaiola Maremmana	37/37	34/34	-	-	Yes
Mora Romagnola	100	864/864	1063/1063	1227/1227	Yes
Nero Siciliano	900/900	1889/1899	3614/3614	4336/4336	Yes
Sarda	-	424/424	566/566	363/363	Yes
Siciliano	100/500	-	-	4950/4950	Yes
Apulo-Calabrese	22	479/479	2159/2159	3556/3556	Yes

Anyway, because of their small populations' size and their typical meat quality traits, the genetic management of the Italian local breeds aims to maintain the original genotypes without any selective project, as it is well declared in the aim of the Pedigree Register. Up today, it is still necessary to adopt mating management programs able to increase the genetic variability and to reduce the inbreeding. However, Nero Siciliano and Sarda populations, presented the opposite issue. Along the years, these two breeds have maintained large populations and the uncontrolled mating led to a very high variability. Likely, in these breeds, the definition of morphological standards and the recovery of an appropriate genotype it would be desirable to fix the typical traits. Similar problem exists, for instance, in Corsican population which shows a high variability because of the uncontrolled mating as consequence of free-range rearing (Casabianca *et al.*, 2000).

2. Breeding systems and product quality

Figure 10. Mora Romagnola pig

The type of breeding system deeply affects meat quality traits, mainly because of the several feeding sources, feeding strategies and animal managements that can be applied among different rearing systems. Furthermore, the choice between selected breeds, native breeds or crossbreeds can also have a great impact on meat characteristics. According to Bonneau and Lebret (2010), the eating quality of meat obtained from crossbreeds is generally intermediate between that of native breeds and that of selected breeds. The differences observed between native breeds and selected breeds, instead, is much more marked. This differentiation is mainly attributable to a combination of factors, such as the genetic effect, the feeding regimen and the rearing system. Indeed, in Europe, native pig breeds generally share very similar rearing systems and some genetic traits that together, confer to their meat well-established characteristics (Pugliese and Sirtori, 2012).

From the genetic point of view, local pig breeds show slow growth rates, reaching the slaughter weight at an advanced age. This implies a greater maturity of the meat that, containing a greater content of myoglobin, shows higher redness and lower lightness (Čandek-Potokar *et al.*, 2003; Pugliese *et al.*, 2005; Teixeira and Rodrigues, 2013; Lebret *et al.*, 2015). Also, the collagen content increases according to the age of animals at slaughter, negatively affecting texture parameters, especially for hardness and cohesiveness. However, some authors observed that the greater fattiness and proteolysis related to physical activity in local pig breeds reared in extensive systems, partially counterbalanced the worsening in texture (Soto *et al.*, 2009, 2010; Lebret *et al.*, 2015). As mentioned above, another characteristic shared by local breeds is a greater potential for lipid deposition either subcutaneous fat then, applying proper feeding strategies, intramuscular fat (IMF). Eventually, these breeds are observed to depot greater amounts of monounsaturated fatty acids, especially oleic fatty acid, than selected breeds (Edwards, 2005).

Despite of their strengths, the use of local breeds for pig production is still very costly. The lack of genetic selection, on one side has allowed the preservation of genetic biodiversity and of some positive traits well-suited to be exploit in the traditional Mediterranean silvo-pastoral systems; on the other side, the uncontrolled mating and the lack of genetic selection has determined the persistence of negative traits, especially linked to reproduction performances (Franci and Pugliese, 2007; Bonneau and Lebret, 2010). Moreover, their slow growth and poor feed efficiency, should be considered in the economical evaluation. Similarly, even if the fat accumulation can positively affect meat quality, fat is considered negatively by consumers and the excessive adiposity rises health concerns. Besides the genetic effect, one of the most important factors affecting quality traits is the feeding, considered either as feeding sources than as feeding strategies. Native breeds are generally reared outdoor, but the modalities and the intensification level of each system are greatly variables. Rearing systems range from the total feed supply through concentrate supplementation, to more extensive systems, where the fattening phase is carried out using only natural resources available in the environment (Pugliese and Sirtori, 2012). In the latter situation, the alimentation plays a pivotal role in determining meat quality traits, indeed, the finishing takes place during autumn in forests of oak or chestnut (Figure 11) and the animals convert large quantities of acorns or chestnuts into fat deposits (Edwards, 2005). The high consumption of acorns or chestnuts, which are rich in starch, induces a compensatory growth response characterized by a very high lipid accretion at both whole-body and intramuscular levels (Lopez-Bote, 1998; Bonneau and Lebret, 2010). This allows pigs, especially in the case of local breeds, to express their high genetic potential for IMF deposition, with subsequent positive consequences on the eating quality of pork and pork products (Lebret, 2008; Bonneau and Lebret, 2010). Moreover, acorns and chestnut are very rich in linoleic and oleic fatty acids,

Figure 11. Typical "Dehesa" pasture. Extremadura, Spain

whereas grass pasture is an important source of n-3 polyunsaturated fatty acids and both α-tocopherol and γ-tocopherol. These latter compounds perform an antioxidant action on lipids (Nilzén *et al.*, 2001; Högberg *et al.*, 2002; Lebret, 2008; Soto *et al.*, 2008; Pugliese *et al.*, 2009; Martins *et al.*, 2018). As a consequence, the backfat and IMF compositions of local breeds reared in extensive systems, is usually characterized by increased proportions of unsaturated fatty acids and decreased proportions of saturated fatty acids (Muriel *et al.*, 2002; Lebret, 2008; Soto *et al.*, 2009). Several authors outlined the importance of the feeding regimen, especially during the finishing phase, in determining the characteristics of the fat and the muscle tissues of local pig breeds (Pugliese *et al.*, 2004, 2010; Rey *et al.*, 2006; Teixeira and Rodrigues, 2013).

Though, an excessive inclusion of herbage in finishing diets might negatively affect the meat quality traits related to manufacturing, but also texture parameters. Studies suggest that multiple factors concurring in the worsening observed for texture. For instance, the lower nutritional value of herbage determines slower growth rates, slower protein turn-over in muscles and lower proteolytic activity; whereas the older age at slaughter leads to a greater content of collagen in meat, increasing the hardness and the cohesiveness (Edwards, 2005). Concerning manufacturing issues, they are generally related to an excessive amount of unsaturated fatty acids in meat. Indeed, even if coupled with antioxidant compounds such as α-tocopherol and γ-tocopherol, the increase of unsaturated fatty acids contents leads to a higher risk of lipid oxidation and, consequently, to the onset of rancid flavors and odors in meat products, especially for products that undergo long storage or curing processes (Nilzén *et al.*, 2001).

The interaction between genetic, environment and rearing system makes local breeds' meat of higher eating quality compared to conventional genotypes. This was, for instance, demonstrated for the traditional British breeds Gloucester Old Spot, Tamworth and Berkshire, for both fresh pork and processed products prepared from the French local breeds Basque, Gascon, Limousin and Blanc de l'Ouest and, of course, the superior quality of the Iberian pig for processing is also well-established (Bonneau and Lebret, 2010). Most of the systems that use local breeds are primarily aimed at the production of high value-added processed products, mostly dry-cured ones. Often, these products are also labelled, or involved in processes for the obtainment of the Protected Designation of Origin (PDO) label, to improve their added value and, thereby, to contribute to the sustainability of these specific production systems and pork chains.

In conclusion, regardless the rearing system, native pig breeds have maintained characteristics such as slower growth rates and greater fatness than conventional breeds (Edwards, 2005), that perfectly match with the traditional Mediterranean silvo-pastoral system, based on limited natural resources, extensive pasture and specific feed sources as acorns and herbage. Contrariwise, commercial breeds have been selected for their fast growth rates and leaner meat; their high nutritional requirements make their employment in free-range low-input systems at least meaningless, if not disadvantageous.

3. Cinta Senese pig breed

Figure 12. "Buon Governo", Ambrogio Lorenzetti. Sala dei Nove of the Palazzo Pubblico of Siena, 1338 - 1339

The first documented proof of the existence of Cinta Senese breed on the Tuscan territory is a fresco of Ambrogio Lorenzetti named "Buon Governo" (Figure 12) and located in the Sala dei Nove of the Palazzo Pubblico of Siena. Cinta Senese is the most numerous Italian native breed in terms of registered animals. It has been registered in the national herd-book for the first time from 1936 to 1966 and again in a regional herd-book since 1976. A very narrow bottleneck was experienced in the 1980's for Cinta Senese due to the low number of animals registered per year (3.6) and of founders (29); this led to a high inbreeding level that, still today, requires an accurate management of the reproducers. In the last decades, the breed underwent an intense recovery program that dramatically increased the number of animals, whereas the inbreeding coefficient diminished from 0.21 in 1996 to 0.17 in 1999 and 0.14 in 2003 (Franci and Pugliese, 2007) (Table 3). The breed is still reared following the traditional free-range system with fattening in woods; the strong presence in the Tuscan territory, the peculiar feeding strategy and management system conferring the meat specific quality traits, have earned the breed a PDO label on fresh meat in 2012. The official reproducers, listed in the anagraphic register, were 131 boars and 809 sows in 2016 (http://www.anas.it/, accessed on September 4th, 2018) and, up to date, 140 farms

(mainly located in the province of Siena) and about 5000 animals can be identified (http://www.cintasenese.org, accessed on September 4th, 2018).

Figure 13. Cinta Senese sow and piglets

Most of the breeders are part of the Consortium of Protection, adhering to the PDO disciplinary. Cinta Senese is a medium size pig, the weight ranges from 250 kg in sows (Figure 13) and 300 kg in boars. Its name "Cinta" derived from the characteristics white band that surrounds the trunk at shoulder level and includes the forelimbs, while the remaining coat is black. The forelimbs' hoofs are white, while the rear ones are black. The head is medium sized, slightly elongated, ears are directed forward and down. In the female, the breasts must be not less than ten, regularly spaced, with normal nipples. Almost the total of the breeders leads either the reproduction than the growing and fattening phases (Figure 14 and 15) in their farms, with very few cases of separation between the reproduction and the fattening activities. Approximately 40% of the farms have 6-15 sows, 30% have between 1 and 5 sows, 20 % of the farms rears 16-50 sows and the rest has more than 50 sows, with few cases of 100 sows and more (Bonanzinga *et al.*, 2012). In half of the farms the weaning takes place averagely at 60 days and 10 kg, for the remaining 50%, 20% of breeders weans at 76 days or more, whereas 30% of them weans at 30 days. Sows give birth to averagely 7 piglets of 1.2 kg twice a year, 6 of them generally survives the weaning (Regione Toscana, 2011). The growing stage (372 g/day) as well as the early, middle, late and overall

Figure 14. Cinta Senese growing pigs

fattening stage is generally characterized by slow growth (439, 334, 322 and 412 g/day) (Unpublished data). These phases generally take place outdoor, farms provided with pasture and woods use them along the entire cycle. The feeding regimens differ from one farm to another, some keep animals outdoor, but the entire ration is supplied by farmer as concentrate; in others farms the level of integration of pasture natural resources varies. Eventually, some farms with large extension of pasture and woods do not adopt any type of integration. This diversity in feeding management results in a great variability of the age and the weight at slaughter (Bonanzinga *et al.*, 2007). The ideal slaughtering weight for this breed is considered 150 kg of live weight. Lighter animals are generally too young, with meats with inadequate maturity, while surpassing the 150 kg often leads to an excessive overall fatness of the carcass. Animals reared indoor or outdoor with plenty of supplementation reach the ideal slaughter weight at 365 days. When feed integration is reduced or absent, animals are slaughter at an average age of 530 days. Also, some variations of the slaughtering weights are registered when farms conduct the finishing phase exploiting the production of chestnut and acorns in wood pasture. In these cases, to take advantage of the exceptional quality traits conferred to the meat by these feeding sources, slaughter might be slightly anticipated or postponed.

Figure 15. Cinta Senese fattening pigs

Table 3. Cinta Senese population from 1991 to 2016

Year	1991	1992	1994	1996	1998	2000	2002	2005	2007	2009	2011	2013	2014	2016
Population Min / Max	77 / -	100 / -	80 / -	182 / -	100 / 500	100 / 1000	1603	2855	2701	2152	2582	958	2613	50000
Trend	-	dec	dec	inc	un	un	un	inc	inc	dec	incr	-	inc	-
Based on	-	census at breed level	-	-	-	census at breed level	census at breed level	-	-	census at breed level	census at breed level	-	census at breed level	-
Breeding Males / Females	7 / 70	8 / 50	10 / 60	22 / 160	- / -	- / -	97 / 348	213 / 994	167 / 895	443 / 1709	153 / 968	123 / 835	131 / 895	131/ 809
Females registered in herdbooks	-	50	46	-	-	-	-	-	-	-	-	-	895	809
Females bred pure	-	40	100	20	-	-	-	-	-	-	-	-	-	-
Herds	-	10	-	-	-	-	-	-	-	-	154	-	-	96
Reliability	un	un	un	un	un	rel	un	un	un	rel	rel	-	-	-

Abbreviations: dec, decrease; inc, increase; un, unknown; rel, reliable

3.1.Nutritional requirements and management systems

Almost all the farms rearing Cinta Senese pigs adhere to the PDO disciplinary that states specific rules for feeding management. According to them, after the fourth month of age, during which the piglets can receive daily food supplementation, the animals must be reared in extensive conditions. The permitted daily feed supplementation cannot exceed 2% of live weight and, at least the 60% of the ingredients must come from the geographical area of production. Moreover, the use of soybean is forbidden, even if this part of the disciplinary is currently under review. Consortium have opened to the employment of soy in the growing phase, since this should not affect the quality of the final products thanks to the fast fat and lipid turn-over. The introduction of soy during growing and for sows, would also help to better fulfill animal's requirements. On the fattening and finishing periods, pasture on wood is carried out in more than half of the farms. The fattening must be done outdoors, but various degree of "extensification" are permitted.

Cinta Senese, as most of the local pig breeds, shows a slow growth rates and a high predisposition to fat deposition, that result in specific nutritional requirements. Few studies are available on growth performances and slaughter traits of Cinta Senese. During fattening and finishing, the average feed intake ranges from an average intake of 2.4 kg/animal/day (Acciaioli *et al.*, 2002) up to 2.8 kg/animal/day (Sirtori *et al.*, 2011). Comparing Cinta Senese and Large White slaughter traits, the former showed about 11% more of fat cuts when reared indoor, this gap decreased by about 3% when the animals were reared outdoor and fed woodlands pasture (Franci *et al.*, 2003). The grater carcass fatness also affected backfat thickness that was higher in Cinta Sense pig compared to Large White ones (Franci *et al.*, 2005). A great variability of feeding regimens is observed among farms. The level of concentrate integration and the availability of acorns, grass and chestnut, make the establishment of a "typical" diet employed for each stage difficult. The available studies have generally investigated together the growing, fattening and finishing phases, considering also that, after the post-weaning phase, most of the breeders uses the same feeding regimen. Commonly, feed concentrate supplied from 50 to 150 kg of live weight, contains about 13-16% of CP, while grass pasture averagely 3.5% of CP (Acciaioli *et al.*, 2002; Campodoni *et al.*, 2003; Sirtori *et al.*, 2011). It is clear how much the level of concentrate provided by the farmers can

influence the diet nutritional value, but before establishing the more appropriate level of integration, the specific nutritional requirements of the breeds, especially in terms of crude protein, needed to be studied. Sirtori *et al.* (2010, 2014), testing four diets with increasing crude protein (CP) contents (8, 10, 13, 16%) in pigs from 50 to 145 kg, observed that the lowest CP level negatively affected the growth performances and the slaughter traits of the animals. Indeed, the group fed 80 g/kg of protein resulted in the lowest slaughter weight and the highest carcass fatness. So, considering the whole period from the post-weaning to the slaughter, a content of 10% of CP diet resulted adequate to fulfill Cinta Senese protein requirements without affecting slaughter and meat quality traits. However, growing and fattening phases should be studied separately to better understand the protein requirements of this breed, also considering that during the growing phase, the ADG is still elevated. Similar studies on Iberian pigs have showed that the ideal protein level with *ad libitum* feeding, is close to 12.9 g of CP/kg of DM from 15 to 50 kg (Nieto *et al.*, 2002) and averagely 9.5 g of CP/kg from 50 to 100 kg of body weight (Barea *et al.*, 2007). The same authors, in a meta-analysis study, have observed how the Iberian pigs have a different pattern of carcass components relative growth, compared to lean and conventional genotypes (Nieto *et al.*, 2014). This confirmes the need to better understand the nutritional requirements in all the stages of growth of local pig breeds, that are clearly different to the ones determined for selected breeds.

3.2. Meat and processed products quality traits

As all the local pig breeds, Cinta Senese meat is characterized by the combination of the genetic and the rearing effects. On fresh meat, studies on Cinta Senese pigs slaughtered at averagely 140 kg, reported that the pH measured in *Longissimus lumborum* muscle at 45 min and 24 h post mortem, was on average 6.39 and 5.69, respectively. Texture parameters showed a greater hardness of Cinta Sense fresh meat than Large White, while water holding capacity resulted lower in Large White animals. Likely, texture was affected by the age and the greater content of collagen related to it, indeed, Cinta Senese pigs reached the slaughter weight at an older age than Large White (Maiorano *et al.*, 2003; Franci *et al.*, 2005). The IMF content was averagely 4% and color parameters, according the CIELAB scale, range from 45.52 to 50.12 for L, from 11.40 to 12.29 for

a* and from 3.04 to 4.62 for b* (Pugliese *et al*., 2005; Franci *et al*., 2007; Sirtori *et al*., 2011). According to several studies on local breeds, Cinta Senese meat resulted darker and redder than Large White one, as suggested by Chroma results (Franci *et al*., 2005).

Another important meat trait is the fatty acids composition, especially considering that, this parameter, is the most affected by the feeding strategy. Indeed, the backfat of Cinta Senese pigs reared indoor, consisted of averagely 36.07 % SFA, 53.3% MUFA and 11.00% PUFA, while animals reared outdoor, with access to pasture, showed a higher content of MUFA (55.08%) and PUFA (13.02%) and a lower content of SFA (31.40%). Despite the higher content of unsaturated fatty acids, the great fatness of primal cuts deriving from local breeds as Cinta Senese, makes their fresh meats less attractive to consumers. Moreover, the higher content of unsaturated fatty acids is mainly due to MUFA and PUFA n-6, whereas PUFA n-3, the healthiest ones, still consisted of 0.5-1%, regardless of the rearing systems. Fatty acids composition is greatly affected by the latest fattening stage, indeed, substituting concentrate with chestnut 3 months before slaughtering, significantly affected meat and fat quality (Pugliese *et al*., 2013). The authors observed that, even if chestnut worsened some physical characteristics of the lean (lower WHC and higher shear force), it increased the intramuscular fat and led to a greater unsaturation level of the adipose tissue, especially with respect to n−3 and n−6 PUFA. From the dietetic point of view, this latter result could be positively evaluated, but a high level of unsaturation, has usually negative consequences on the technological aspects. Though, this was not the case, indeed, the low level of malondialdehyde (MDA) observed, suggested a protective effect of chestnut for lipid oxidation. Likely, the high levels of α-tocopherol in acorns and γ-tocopherol in chestnut, could have acted as antioxidants, as observed also in other studies of the same authors (Pugliese *et al*., 2009, 2010).

Manufactured products currently represent the most important market for Cinta Senese farmers. A great variety of products are available, i.e. dry-cured ham, salami, Pancetta, Lardo, Capocollo and many others cured products. As for the fresh meat, extensive breeding, if practiced with a rational exploitation of forest resources (acorn and chestnut), can lead to the development of favorable aromas, such as esters and aldehydes in dry-cured ham (Pugliese *et al.*, 2009) and, therefore, to products with excellent sensory properties. For instance, the cured Lardo of chestnut and acorns-fattened pigs

resulted in the best organoleptic scores (Sirtori *et al.*, 2005). However, another study on the same product showed an interesting result: panelists underscored the appearance-related traits of chestnut and acorn products (Pugliese *et al*., 2010). Authors assumed that consumers have associated the higher yellowness and oiliness of these products to rancidity signs and not to the inclusion of chestnut and acorns in the finishing diet. This confirms that Cinta Senese products are still niche products, most of the potential consumers are not aware of their peculiarities and of the interaction between the rearing system and the sensory characteristics of the resulting products.

As fresh meat has a limited market, most of the carcass that cannot be used to be directly cured (Lardo, Ham, etc…), is employed to produce transformed products. For instance, burgers of Cinta Senese meat are slowly spreading in the local supermarkets, as well as dry or semidry fermented salami and sausages that are widely appreciate by consumers and, likely, the most known products of this breed. Salami and dry-fermented sausages, according to the Southern Europe tradition, are generally characterized by slowly air-drying and mold-ripening (Flores, 1997). This curing process leads to peculiar characteristics and flavors that are widely appreciated by consumers. Natural fermentation is usually adopted, but without the addition of lactic acid-producing starter cultures, the risk of developing harmful bacteria, such as *Listeria monocytogenes* or *Clostridium botulinum* increases (Lücke, 2000). Thus, to avoid a severe deterioration of nutritive and organoleptic attributes due to lipid oxidation, as well as to ensure food safety, several synthetic food preservatives are commonly included. Among them, the most used are nitrites and nitrates (Hammes, 2012). These compounds positively affect color, inhibit the growth of pathogenic bacteria, contribute to the development of the typical cured meat flavor and delay oxidative rancidity (Marco *et al.*, 2006). Despite their effectiveness as curing agents, the nitrite/nitrate intake represents a risk for human health, i.e. the formation of carcinogenic nitrosamines is one of the most recent concerns (De Mey *et al*., 2017).

4. Dealing with novel issues for the pork chain: animal welfare, healthier products and environmental impact

Enlarging the market for Cinta Senese meat and cured products, necessary should pass through initiatives to educate consumers to their characteristics. Though, pork chain has

also to attend consumer's demand that are currently changing towards healthier products and a greater attention for environmental and animal welfare issues (Krystallis *et al.*, 2009).

4.1. Healthier products

In the last decades, following the increasing demand for healthier meat products, research has focused on lowering fat and salt content of meat and processed meat products and on finding alternatives to chemical preservatives (Shan *et al.*, 2017). The link between meat consumption, the onset of cardiovascular diseases and some type of cancer is widely known. Indeed, in 2015, the International Agency for Research on Cancer classified processed meat as "carcinogenic to humans" (IARC Monographs, 2018).

In pork chain, the genetic selection has led to leaner meat, however, the natural content of SFA and n-6 PUFA is still high. Accordingly, the recommended ratio of PUFA to SFA is between 0.4 and 1.0, and the n-6/n-3 PUFA ratio should not exceed 4 (Garg *et al.*, 2006; Jiménez-Colmenero, 2007). Diets excessively rich in n-6 PUFAs and with a high n-6/n-3 PUFA ratios promote cardiovascular disease, cancer and inflammatory and autoimmune diseases, whereas increased levels of n-3 PUFAs (and low n-6/n-3 PUFA ratios) exert suppressive effects (Simopoulos, 2002). So, lowering the fat contained in meat is not enough to ensure an enhancement in healthiness, also the qualitative aspects of fat need to be considered. Western diets are far below the recommended minimum of 250 mg per day of long-chain omega-3 PUFAs (Sanders, 2000); moreover they contain excessive amounts of n-6 PUFAs, with an n-6/n-3 PUFA ratio of 15:20 as opposed to the recommended range of 1:4 (Simopoulos, 2002). Among long-chained n-3 PUFA, alpha-linolenic acid (ALA C18:3 n-3) and its longer-chain metabolites, i.e. eicosapentaenoic acid (EPA C20:5 n-3) and docosahexaenoic acid (DHA C22:6 n-3), play an important role in human health (Pelliccia *et al.*, 2013; Bos *et al.*, 2016). The main sources of EPA and DHA are oily fish, oil fish supplements and, to a lesser degree, white fish and shellfish, which are hardly appreciated in most Western countries. Consequently, many studies focused on improving the nutraceutical quality of widespread foods by increasing their omega-3 content. Meat and meat products are an interesting target because of their large consumption and their fatty acid profile. Indeed,

they are characterized by a low content of long-chain n-3 PUFA together with a high presence of MUFA (approximately 45-50%) and SFA (approximately 45–55%) (Givens *et al.* 2006). The type of food selected for the enrichment should be well accepted, popular, inexpensive and easy to cook, according to the changing diet habits and the increasing demand for "ready-to-eat" products, such as burgers.

Besides the fat, the other great concern for human health is the use of chemical preservatives in processed meat products. Several studies have been focusing on nitrate/nitrite reduction or substitution (Purriños *et al.*, 2013; Özvural and Vural, 2014; Pateiro *et al.*, 2015), but the main issue remains finding an alternative able to address the multiple activities they perform. Nitrite and nitrate remove moisture and reduce the water activity, perform antimicrobial activity mainly against *Clostridium botulinum* and *Listeria monocytogenes* and concur to develop several distinctive characteristics of cured meat products, such as red color, texture and cured flavor. Eventually, they exert a protective action against oxidative processes (Alahakoon *et al.*, 2015). Up till now, most of the alternatives proposed are plant extracts, largely obtained from agricultural by-products. These compounds are very rich in polyphenols, flavonoids and terpenoids, that are able to perform a double antioxidant-antimicrobial function (Falowo *et al.*, 2014; Hygreeva *et al.*, 2014; Shah, *et al.*, 2014). These compounds might constitute also a great opportunity to exploit agricultural by-products, which otherwise would be wasted.

4.2. Environmental impact and animal welfare

The intensification of production systems in agriculture and the increase of efficiency taking place in the last century, has led to unwanted environmental consequences and compromised animal welfare. After the 70's, consumers' awareness for these topics raised, pressing for the adoption of more sustainable agricultural practices. Extensive research has been carried out on pig production systems and it involved strategies to combine system's sustainability, economic profit, social and environmental goals (Krystallis *et al.*, 2009). After poultry, pig production occupies the second place on consumer's concerns about animal welfare (Martelli, 2009). Generally, outdoor rearing systems are favorably perceived by consumer (Eriksen *et al.*, 2006), who associated them with most of the main ideas linked to the concept of welfare, i.e. "space allowance", "access to outdoor areas" and "exposure to natural light", "absence of movement

restriction by chains or tethers", "expression of natural behaviors" and "social contact" (Martelli, 2009). Besides consumer perception, the outdoor housing leads both to beneficial and adverse effects on pig welfare. Animals kept outdoor on paddocks and pasture can express their natural behavior (locomotion, rooting etc…) easier and on a larger scale than indoor pigs. On the other side, the lesser control performed on housing conditions increases the risk of occurrence of parasites, injuries and diseases (Millet *et al.*, 2005). Moreover, the exposure to climatic extremes can negatively affect feed efficiency, causing greater excretion of unutilized dietary nutrients. In summer, losses of nitrogen by gaseous emission can be high and, when autumn rainfall occurs, losses by leaching can also be substantial (Eriksen and Kristensen, 2001; Eriksen *et al.*, 2006; Halberg *et al.*, 2010). In organic systems, where total nutrient loading of land is regulated, and in Mediterranean systems, where stocking density is much lower, and woodland and pasture are maintained, environmental impact is less severe and a better nutrients recycling within the production system is achieved. In these latter systems, pigs also plays a central role in forest management and Mediterranean landscape conservation (Edwards, 2005).

In conclusion, Mediterranean silvo-pastoral systems, already incorporate a great number of positive features that have a great potential to enlarge the market of these pork products, addressing consumers' demand for eco- and animal-friendly products, but also match the increasing demand for natural and traditional products. For these systems, the current challenge is deal with the increasing demand without losing their specific characteristics, that mark the difference between them and the intensive production systems from which consumer are moving aside. Knowledge on local pig breeds should be improved and the best management practices should be outlined in order to maximize their reproductive and growth performances without losing their genetic biodiversity. Their specific metabolic patterns in lean and fat deposition should be considered to develop appropriate feeding strategies, able to improve meat eating quality and, at the same time, to reduce the environmental impact by avoiding nutrient wastes and nitrogen excretions. Lastly, besides the great appreciation towards meat and meat products belonging to local pig breeds and free-range systems, market evolution cannot be ignored. The rising attention towards health, requires an attempt to innovate traditional pork products with novel and healthier ingredients, without losing their identity.

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AIM

The aim of this thesis was to improve the knowledge on Cinta Senese pig breed and to enhance its performances and the quality of its products. Local pig breeds are an untapped resource for pork market, but the economic convenience of their rearing is still low and Cinta Senese farmers are struggling to meet the increasing consumer demand for traditional and healthier products. Research on Iberian breed have shown that local breeds need specific feeding and management strategies to optimize their performances without losing the well-known quality of their meats, linked to their traditional rearing systems. The success of Iberian products worldwide, has also shown that an appropriate educational strategy for consumer is needed. Sensory quality itself is not enough to enlarge the market of local breed products, but consumers need to understand what they are buying and what they are paying for. Consumers demand for eco-friendly products, as well as the demand for healthier products are important issues of today's society. To enlarge and to make more competitive the Cinta Senese production chain, addressing these two issues is of central importance. Cinta Senese mainly produces fresh meat and dry-cured products, with the seconds being the most known and appreciate by consumers. The fresh meat's market is facing the challenge to make appetible a product that is noticeably fat, even if, being mostly fattened at pasture and fed chestnut and acorns, Cinta Senese meat is richer in MUFA if compared to the meat deriving from most of the intensive pork production systems. On the other hand, the consumption of cured products is rising health concerns due to the employment of nitrite and nitrate as curing agents.

This work developed in two main objectives:

Trial 1. The knowledge on Cinta Senese nutritional requirements is still limited to a global approach on the overall rearing period, but, as it is largely known for commercial pig breeds, each stage of growth has specific requirements, whose adequate fulfillment has positive effects on the end products' quality. During the growing stage, the feeding has limited effects on meat composition, due to the lipid and the protein turn-over, but it deeply affects the carcass yield and the ratio of fat and lean deposition during the whole animal life. Hence, improving the knowledge on the protein requirements for this phase, will help farmers to provide the best dietary formulation to Cinta Senese growing pigs,

enhancing their *in vivo* performances and slaughtering traits. Besides the economic saving for the farmers, feeding a diet with an adequate protein content has also important consequences on the environment. Indeed, the outdoor rearing systems required by the PDO disciplinary, has been linked to an increased risk of nitrogen leaching, since nitrogenous excretions are exposed to atmospheric events. Therefore, provide the adequate protein intake to maximize animal performances without increasing environmental pollution meets a current topic for pig and livestock production.

Trial 2. In the present thesis, two possible innovations were tested to meet consumer's demand for healthier products and, at the same time, to preserve the eating quality and the traditional recipes linked to Cinta Senese production. The feasibility to improve the fatty acid profile of Cinta Senese meat was tested on a largely appreciated fresh product: the burger. The proposed innovation dealt with the addition of fish oil to burgers. Fish oil is very rich in long-chained omega-3 PUFA, EPA and DHA, that are demonstrated to be very beneficial to human health. These fatty acids are largely present in fish products, but absent in meat. The enrichment was carried out in two different ways, by adding bulk fish oil and microencapsulated fish oil to assess the better technique to maintain product's quality traits and to preserve the added PUFA from lipid oxidation. Among the cured products, a type of dry-fermented sausage was chosen to test two different mixtures of vegetal extracts with antimicrobic and antioxidant properties as potential replacement for sodium nitrite. The effectiveness of introducing this innovation in this class of products was tested by evaluating the effects on the aromatic profile, lipid oxidation, microbial microflora, sensory characteristics and consumer willingness to pay for the new products.

MATERIALS AND METHODS

Trial 1. Protein requirements

1. Diets

The tested diets contained four protein levels: 120, 140, 160 and 180 g/kg as fed basis (named CP12, CP14, CP16 and CP18, respectively) and had the same protein quality in terms of balance in essential aminoacids. Diets formulation was made according the Nutrient Requirements of Swine (NRC, 2012), considering, as reference, protein and AAs requirements of pig of 50 kg (16% of CP as feed) and modifying the other diets considering the AAs balance, according the ideal protein composition. Table 1 shows ingredients and chemical composition for each dietary formulation. Diets were pelleted to avoid single ingredients to be selected by the animals. Animals were fed twice a day (at 8.00 am and 4.00 pm in equal meals). Two percent of bentonite was added to each formulation to increase the Acid Insoluble Ash (AIA), used as internal marker to assess diet's digestibility.

Table 1. Ingredients and chemical composition (% d.m.) of diets

		Diet			
		CP12	**CP14**	**CP16**	**CP18**
Ingredients					
Maize		73.50	68.00	62.95	57.40
Soybean meal (46%)	%	9.00	14.50	19.50	25.00
Wheat bran	"	10.00	10.00	10.00	10.00
Maize oil	"	2.00	2.00	2.00	2.00
Bentonite	"	2.00	2.00	2.00	2.00
Lysine HCl		0.45	0.45	0.45	0.50
Methionine		0.05	0.05	0.10	0.10
Premix[1]	"	3.00	3.00	3.00	3.00
Composition					
Dry matter	%	87.92	88.18	88.16	88.07
Crude Protein	"	13.34	15.58	17.86	20.37
Ether extract	"	4.87	4.71	4.73	4.76
Crude fiber	"	5.08	4.03	3.57	4.64
NDF		19.91	18.89	19.38	17.69
ADF		6.60	7.37	7.63	8.61
ADL		1.46	1.79	2.11	1.99

Table 1. Ingredients and chemical composition (% d.m.) of diets

		Diet			
		CP12	**CP14**	**CP16**	**CP18**
N-free extract	"	69.90	68.11	66.15	62.55
Ash	"	6.81	7.58	7.69	7.68
Lysine[2]	"	0.99	1.15	1.30	1.46
Methionine[2]		0.31	0.34	0.42	0.44
Gross energy[3]	Mj/kg	18.296	18.270	18.402	18.570

[1]Premix provided per kg of feed: Vitamin A: 13500 IU; Vitamin D3: 1200 IU; Vitamin E: 12 mg; Vitamin K: 2.25 mg; Vitamin B1: 2.4 mg; Vitamin B2: 4.8 mg; Vitamin B6: 1.8 mg; Vitamin B12: 0.024 mg; Niacinamide 24 mg; Calcium pantothenate: 1.35 mg; Folic acid: 0.3 mg; Choline HCl 495 mg; Iron(II) carbonate 90 mg; Copper(II) sulfate 93 mg; Manganese oxide 30 mg; KI 1.96 mg; Na Selenite 0.3 mg; DL Methionine 105 mg; L Lysine 60 mg; [2]Calculated from tabulated data of the ingredients; [3]Calculated from specific values of the analytical components

2. Animals

A total of 38 castrated males of Cinta Senese pigs were employed. Twelve animals were bought from Azienda Agricola Borgonovo (Siena, Italy) to be used in the first replication of the feeding test. The remaining 26 pigs were bought from the Azienda Agricola Rosa dei Venti (Pisa, Italy); twelve of them were used for the second replication of the feeding test, 8 pigs were used for the digestibility test, while the last 6 pigs were immediately slaughtered. Table 2 gives an overall insight of the experimental design.

2.1. In vivo performances and slaughtering traits

Twelve Cinta Senese castrated males, of 28 kg of live weight and 135-days old on average, were divided in 4 dietary groups and allocated in individual pens provided with automatic nipple for water and chains according to animal welfare requirements. Individual boxes were divided in three blocks of 4 boxes in the barn were the trial took place and inside each block every dietary treatment was tested. The trial was repeated twice for a total of 24 pigs and 6 animals for each diet. The feeding level adopted was 0.95 x *ad libitum* and troughs were refilled thrice a day. Feed was stored separately for each individual pen in order to weekly calculate the feed consumption by weight's difference between one week and the following one. The first and the second replications lasted 6 and 7 weeks respectively, when animals reached the average target weight of 65 kg and they were slaughtered together to determine the carcass composition, tissues

distribution and nitrogen content. Six more pigs were slaughtered at the beginning of the first trial, at the average weight of 28.8 kg; in line with the initial live weights of the 24 pigs in trial. They were used to estimate the initial carcass composition and nitrogen content of the pigs in trial, according to the comparative slaughter method. After slaughtering, the two half-carcasses of each animal were weighed separately, cooled for 12 hours and then the right side was dissected in the anatomical cuts: head, neck, shoulder, ribs, loin plus belly, and ham, all comprehensive of the surrounding subcutaneous fat and skin. The main cuts were weighed and then divided in subcutaneous fat plus skin, intermuscular fat, lean and bone. Tissues were weighed separately and sampled to be analyzed, whereas bone was discarded. Subcutaneous fat, intermuscular fat and lean (Longissimus dorsi (LD) separately) of each cut were analyzed for proximate composition according the official methods (AOAC, 2012). Total lean, fat and bone of carcass were calculated and the total nitrogen content in lean was also obtained as sum of the individual cuts' composition. Moreover, the gain in lean, fat, bone and nitrogen, during the whole trial was calculated as difference between the final and the initial composition, estimating the latter by the regression on live weight and anatomical cuts of the six piglets slaughtered at beginning of the trial.

Table 2. Experimental design of Trial 1

Animals		**Samples type**		**Sampling**
Individual pens	24 Cinta Senese castrated males (+6 animals immediately slaughtered) Total= 30 animals	*In vivo*	Weight, FDI, ADG, backfat thickness	Once a week
			Carcass: weight and yield	At slaughtering
		Post-mortem	Anatomical cuts (head, neck, shoulder, ribs, loin, ham): weight and tissue composition (backfat, intermuscular fat, lean and bone)	12 hours after slaughtering
Metabolic cages	8 Cinta Senese castrated males	*In vivo*	Weight	Every time animal was put in metabolic cage
			FDI, ADG and urine	Daily at 4.00 pm
			Faeces	Twice a day at 9.00am and 4.00 pm

Abbreviations: FDI (Feed daily intake), ADG (Average daily gain)

2.2.Digestibility and nitrogen balance

Eight Cinta Senese castrated males were divided in two groups of 4 pigs each and they underwent 8 experimental cycles in metabolic cages (60 x 130 cm). Every animal tested all the diets at different ages, according a Latin square design. The trial lasted a total of 9 weeks, animals were 5 months old, they weighed on average 55 kg at the beginning of the trial and 75 kg at the end. The same four formulations previously described in Table 1 were contemporarily tested by four alternating pigs that occupied the four available cages. Animals were fed twice a day (at 8.00 am and 4.00 pm in equal meals). Daily feed allowance was 90 g/kg of metabolic weight. When present, refusal was collected before the morning distribution to calculate the actual intake in the previous day. Cages were supplied with water nipple and trough and were adapted to separately sampling faeces in trays and total urine in flasks containing 20 ml of 8N sulphuric acid to avoid ammonia loss (Sardi et al., 1998). Each cage period lasted 5 days and was preceded by a 9-day period on floor for adaptation to diet. The cage period consisted of a phase of adaptation (2 days) and a phase of faeces sampling and total urine collection (3 days) according to Zhang and Adeola (2017) and in line with other total tract digestibility trials (Nieto et al., 2002; Acciaioli et al., 2011; Wang et al., 2017); the length of cage period was planned to address the ministerial prescription on animal welfare (Authorization n 84/2016-PR – 28/01/2016). Room temperature was set at 21°C and relative humidity was about 80%. Animals were weigheded before each turn in cage to adjust the amount of feeding on their metabolic weight. The collection of total urine and faeces samples took place at fixed hours, twice a day for faeces (9.00 am and 4.00 pm) and once a day for urine (4.00 pm). The daily urine production of each subject was weighed and then a sample of the total urine was taken. Faeces and urine collected in a day were associated to the feed intake of the previous day to calculate digestibility and nitrogen balance. The experimental design was showed in Table 3.

Table 3. Latin square design for digestibility test: adaptation/cage cycles

Week	Group 1	Group 2
1	Box diet A	---
2	Cage diet A	Box diet D
3	Box diet B	Cage diet D
4	Cage diet B	Box diet C
5	Box diet C	Cage diet C
6	Cage diet C	Box diet B
7	Box diet D	Cage diet B
8	Cage diet D	Box diet A
9	---	Cage diet A

Trial 1 was carried out in the facilities of the Department of Agriculture, Food and Environmental Science -DISPAA - Florence.

3. Chemical analysis

Moisture, protein content, ash and ether extract of the feeding trial's samples were determined according to AOAC (2012). The analytical methods used for feed, faeces and urine are reported by Martillotti *et al*. (1987). Dietary formulations and faeces were analyzed for proximate analysis and acid-insoluble ash (AIA). AIA was used as undigestible internal marker to calculate the total tract apparent digestibility (TTAD) of dietary components following the method proposed by Van Keulen and Young (1977).

4. Statistics

Data were analyzed by GLM Procedure (SAS, 2007) using the following model:

4.1. In vivo performances and slaughtering traits

$Y_{ijkl} = \mu + P_i + T_j + B_k + b*X_{ijkl} + E_{ijkl}$

Where

Y = l^{th} observation;

P = fixed effect of the i^{th} Protein content (1, .. 4)

T = fixed effect of the j^{th} trial (1, 2);

B = fixed effect of k^{th} block of pens (1,.. 3).

X = continuous effect of initial live weight of pig;

E = random error.

4.2. Digestibility and nitrogen balance

$Y_{ijkl} = \mu + P_i + S_j + D_k + c*X_{ijk} + E_{ijkl}$

Where

Y = l^{th} observation on j^{th} subject;

P = fixed effect of the i^{th} Protein content (1, .. 4)

S = fixed effect of the j^{th} subject;

D = fixed effect of the i^{th} day of sampling (1, ..3)

X = continuous effect of Metabolic weight of pig at entry in cage;

E = random error.

Statistical differences among mean values were assessed using Student's t test, with the level of significance established at 5%. The dietary formulations were clustered according their CP levels in low protein (LP) formulations (CP12+CP14) and high protein (HP) formulations (CP16+CP18), considering as medium point the CP supplementation usually adopted for selected pig breeds. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of dietary treatment and the significance of difference between LP and HP groups.

Trial 2. Healthier products

1. Burgers

Burgers were made with loins of Cinta Senese pigs provided by Azienda Agricola Borgonovo (Cortona, AR, Italy). Ten pigs were bred outdoors and fed with commercial feed. Ten portions of fresh loins (a total of approximately 11kg of meat) were minced and mixed with salt (2%), sulfites (0.05%) and mashed potato powder (2.4%). Three types of burgers were made (Table 4): control (C) (3.7 kg of mixture), with no further modifications, microcapsule burgers (M) adding 173 g of microcapsules to 3.7 kg of mixture and fish oil burgers (F) adding 6 g fish oil to 3.7 kg of mixture.

Table 4. Experimental design of bugers

Treatment	Total samples	Storage	Samples for chemical analysis	Samples for sensorial analysis
Control (C)	41	T0	10 (5 fresh + 5 cooked)	7 (only cooked)
		T5 – refrigerated at 4°C for 5 days	5 (cooked)	7
		T30 - frozen at -18°C for 30 days	5 (cooked)	7
Bulk fish oil (F)	41	T0	10 (5 fresh + 5 cooked)	7 (only cooked)
		T5 – refrigerated at 4°C for 5 days	5 (cooked)	7
		T30 - frozen at -18°C for 30 days	5 (cooked)	7
Microencapsulated fish oil (M)	41	T0	10 (5 fresh + 5 cooked)	7 (only cooked)
		T5 – refrigerated at 4°C for 5 days	5 (cooked)	7
		T30 - frozen at -18°C for 30 days	5 (cooked)	7

The respective quantities of microcapsules and fish oil were calculated to contain the same amount of EPA+DHA(1.67g). The burgers were made by weighing 90g of mixture and shaping it into a standard burger mould. For cooked samples, the following cooking

procedure was used: grilling at 165 °C, flipping every 2 min until reaching an internal temperature of 73–75 °C, recorded using a thermometer probe (Testo 735-2, Lenzkirch, Germany).

Forty-one burgers were made for each addition (C, M, F) and used as follows: five burgers underwent physico-chemical analysis as fresh matter; five burgers were cooked and used for physico-chemical analyses at 0 days (T0); seven burgers were cooked and underwent sensorial analysis at T0; five burgers were stored at 4 °C for 5 days (T5), then cooked and analyzed; seven burgers were stored under the same conditions (T5), cooked and examined by panelists; five burgers were stored at -20 °C for 30 days (T30), then cooked and analyzed; seven burgers were stored under the same conditions (T30), cooked and examined by panelists.

1.1. Bulk fish oil

Fish oil was kindly provided by Biomega Natural Nutrients (Galicia, Spain). It is a low viscosity, vacuum deodorized oil. As reported by the producer, its omega-3 PUFA contents for 100 g of product are 5.96g of EPA and 25.83g of DHA.

1.2. Multi-layer microcapsules

Multi-layer microcapsules were elaborated following the methodology of Jiménez-Martín *et al*., (2015) with some modifications. Fish oil (Biomega Natural Nutrients, Galicia, Spain) was used as a source of omega-3 PUFA (5.96% EPA, 25.83% DHA, 0.02% BHT). The process started from a primary emulsion made of 20 g of fish oil, 6 g of soy lecithin (Biogran S.L., Madrid, Spain) and dissolved in 174 g of water. This mixture was added to a solution made of 2 g of chitosan (Trades, Chitoclear FG 95, Murcia, Spain) dissolved in 198 g of acetic acid (1%) (Scharlau, Barcelona, Spain) to form a secondary emulsion, which was homogenized (Homogenizer SPX, APV2000, Denmark) at 700bar. This was added to 400 g of water and a maltodextrin (30%) (Roquette, Glucidex 12, Lestrem, French) solution for finally obtaining a feed emulsion. The feed emulsion (800g) was dehydrated and turned into powder using a laboratoryscale spray-dryer (Mini Spray Dryer B-290 Buchi, Switzerland) equipped with a 0.5 mm nozzle atomizer. The aspirator rate was adjusted to 80%, feed rate was 1 L/h, inlet temperature was 180 °C, and outlet temperature ranged 85–90 °C. The obtained

microcapsules contained 9.63 mg of EPA+DHA per gram of microcapsules and 4% moisture, which was calculated following the methodology of Jiménez-Martín *et al.*, (2016).

2. Dry-fermented sausages

2.1. Antioxidant mixtures

The natural antioxidants employed in the present studies were provided by Phytolab (Sesto Fiorentino, Florence, Italy). They consisted of grape seed and chestnut extracts, tocopherol and hydroxytyrosol (extracted by defatted olive pomace). The manufacturer provided the, total phenolic content and antiradical scavenging activity (EC50) of each extract (Table 5). The grape seed and chestnut extracts were combined with the same amount of hydroxytyrosol and tocopherol to form two different mixtures; grape seed (GSE) and chestnut (CHE) mixtures.

Table 5. Total phenolic content and radical scavenging activity of natural antioxidant constituting the mixtures.

	Total phenolic content	**Antiradical scavenging activity (EC_{50})**
Grape seed extract	822.709 (mg/g)	0.147
Chestnut extract	161.091 (mg/g)	0.085
Olive pomace (hydroxytyrosol)	32.62 (g/l)	0.196
α-tocopherol	–	0.184

2.2.Sausages manufacturing

In an industrial plant (Azienda Agricola Savigni, Pistoia, Italy), 24 kg of pork lean and 6 kg of subcutaneous backfat from Cinta Senese pig breed were minced and equally divided in three batches (Table 6). Salt (23 g/ kg), sucrose (35 g/kg) and black pepper (0.2 g/kg) were added to each batch following the recipe traditionally used by the manufacturer. Thirty ppm of sodium nitrite (E250) were added to the first batch to constitute the control (NIT). In second batch, 10 g/kg of GSE mixture were used to replace sodium nitrite, while 10 g/kg of CHE were added to the third batch. Sausages were weighed, dried at 28 °C and RH 85% for 4 days and then ripened 21 days (T 13 °C, RH 70%). Once ripened, six samples of each batch were collected, pH, color, and

processing loss were immediately measured. Samples were vacuum packed and stored at −80 °C for physical, chemical and aromatic analysis. Another 3 samples of each batch were stored at 4 °C to be employed for sensory analysis the following day. This design was replicated to have two totally independent batches for each treatment.

Table 6. Experimental design of dry-fermented sausages

Treatment	Total samples	Curing	Samples for chemical analysis	Samples for sensorial analysis
Sodium nitrite (NIT)	37	28 °C, RH 85% for 4 days 13 °C, RH 70% for 21 days	12	25
Grape seed extract + hydroxytyrosol (GSE)	37		12	25
Chestnut extract + hydroxytyrosol (CHE)	37		12	25

3. Analysis

The analysis performed in Trial 2 are described in detail below and a summary is listed in Table 7.

3.1. Analysis on burgers

Fresh (n=15) and cooked (n=45) burgers were first analyzed by means of instrumental color. Then, samples were minced, and water activity and moisture were immediately determined. The rest of sample was stored at -80 ºC until further analysis. Cooking loss was calculated as the difference between the weight of cooked and fresh burgers and it was expressed as a percentage. Instrumental color was measured on the surface of the burgers. Fresh samples were examined the same day they were processed. In the case of cooked samples, measurements were taken when they had reached room temperature (20–25 °C). Instrumental color was determined using a Minolta CR-300 colorimeter (Minolta Camera Corp., Meter Division. Ramsey, NJ) with illuminant D65, a 0º standard observer and a 2.5 cm port/viewing area. The following color coordinates were determined: lightness (L*), redness (a*) and yellowness (b*). The colorimeter was standardized before use with a white tile having the following values: L*=93.5, a*=1.0 and b*=0.8.

For the water activity, the system Lab Master-aw (NOVASINA AG, Switzerland) was used after calibration.

The moisture in fresh and cooked samples was determined gravimetrically at 100 ± 2 ºC by the official method (AOAC, 2000a, reference 935.29).

Fat content was determined gravimetrically with chloroform:methanol (2:1, vol/vol), following the method described by Pérez-Palacios *et al.*, (2008).

Protein content was calculated in duplicate by the Kjeldahl method (AOAC, 2000b, reference 992.15).

Fatty acid methyl esters (FAMEs) from extracted fat were prepared by basic transesterification following the official method (AOAC., 2000c, reference 963.22), using hexane and hydroxide potassium 2N. FAMEs were analyzed by gas-chromatography (GC) using a Hewlett–Packard HP-5890A gas chromatograph, equipped with an on-column injector and a flame ionization detector (FID), using a polyethylene glycol capillary column (Supelcowax-10, Supelco, Bellefonte, PA, USA) (60 m × 0.32 mm i.d. × 0.25 μm film thickness). The GC oven program temperature was as follows: initial temperature of 180 °C was raised at 5 °C/min to 200 °C, kept at this temperature for 40 min, raised at 5 °C/min to 250 °C, and then kept for an additional 21 min. The injector and detector temperatures were 250 °C. The carrier gas was helium at a flow rate of 0.8 ml/ min. Individual FAME peaks were identified by comparison of their retention times with those of standards (Sigma, St. Louis, MO, USA). Peak areas were measured, and FAMEs were expressed as area percentage of total area FAMEs (%).

Thiobarbituric acid-reactive substances (TBARS) were measured following the extraction method described by Salih *et al*., (1987). Each burger was minced in a kitchen blender, and 2.5 g were homogenized for 2 min with 7.5 mL of 3.86% perchloric acid and 0.25 mL of butylated hydroxytoluene (4.2%). The tubes were kept on ice to avoid heat degradation. This homogenate was filtered and centrifuged (4 min, 3500 rpm). The supernatant (2 mL) was mixed with 2 mL of thiobarbituric acid 0.02 M. At the same time, a standard curve was prepared employing 1,1,3,3- tetraethoxypropane (TEP). Immediately, the mixture was heated to 90 ºC for 30 min, cooled and centrifuged again (2 min, 3500 rpm). Absorbance was measured at 532 nm on a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The concentration of malonaldehyde (MDA) was

calculated from the standard curve, developed simultaneously with the samples using solutions of TEP (Merck, Schchardt, Germany). TBARS were expressed as mg MDA kg-1 sample.

All sessions were done in sensory panel rooms with the conditions specified in the Regulation UNE-EN-ISO 8589:2010 (UNE-EN-ISO 2010) equipped with white fluorescent lighting (220-230 V, 35 W). A piece of cooked burger (20g) was served hot on white plastic plates to panelists, marked with random three-digit codes. The panel sessions were held around 1-2 h before lunch time. Salt-free crackers and a glass of water at room temperature were provided to each panelist to rinse between samples. The burgers were assessed by a trained panel of 18 members using a descriptive analysis method. Eleven sensory traits grouped under appearance (greasy appearance), odor (odor intensity, cooked meat odor) texture (hardness, juiciness, oiliness), taste (salty) and flavor (cooked meat flavor, rancid flavor, flavor intensity, after taste) were assessed. Selected subjects underwent further training in meat and meat products sensory characteristics over five years. The number of burgers used for sensorial analysis was 7 for each supplementation, repeated for T0, T5 and T30. Each burger was divided into four parts to be served to panelists for a total of 28 pieces of burgers of each type (C, M, and F). Each panelist evaluated three pieces of burger in each session, and the sample order was randomized across assessors. Sensory traits were assessed by panelists in a 10 cm unstructured line, ranging from "less" to "more".

3.2. Analysis on dry-fermented sausages

Physical, chemical and microbial analysis

At the end of ripening, physical parameters were assessed on 12 samples of each batch (6 for each replication). Sausage pH was measured at room temperature (20 °C) using a pH meter Crison GLP21 (Barcelona, Spain), the instrument was introduced in a sausage portion. Color (L*, a* and b*) was determined by a Minolta Chromameter CR200 (Tokyo, Japan) immediately after slicing. aw was measured following the method ISO 21807:2004. Two 10 mm-thick and 10 mm-width slices of each sample, were cut and immediately analyzed at room temperature (22 °C), using a Zwick Roell Z2.5 apparatus (Ulm, Germany) with a loading cell of 1 kN at the crosshead speed of 1 mm/s. Texture profile analysis (TPA) was performed assessing the following parameters: hardness,

cohesiveness, gumminess, springiness and chewiness. Moisture was determined by lyophilizing to constant weight 40 g of sample, according to AOAC methods (2012). Weight loss was measured as the difference between weight at time zero and end of ripening (after 24 days). Total protein, fat and ash contents were determined following AOAC (2012) methods. Lipid oxidation was determined according Vyncke (1970), using a PerkinElmer Lambda EZ150 spectrophotometer (Waltham, MA, USA). Results were expressed as mg of malondialdehyde (MDA)/kg of samples. Fatty acids were determined using a Varian GC-430 apparatus equipped with a flame ionization detector (FID) (Palo Alto, CA, USA) as reported by Sirtori *et al*. (2015). The individual methyl esters were identified by their retention time using an analytical standard (F.A.M.E. Mix, C8-C22 Supelco 18,9201AMP). Response factors based on the internal standard (C19:0) were used for quantification and results were expressed as mg/100 g of sample. The fatty acid content was reported as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Microbiological analyses were carried out in an external accredited laboratory to determine the products' safety. The following bacteria were investigated: *Escherichia coli* (ISO 16649–2:2001), *Listeria monocytogenes* (UNI EN ISO 11290–1:2005), coagulase positive *Staphylococcus* spp. (UNI EN ISO 6888–1:2004), *Clostridium botulinum* (ISO 15213:2003) and *Salmonella* spp. (UNI EN ISO 6579:2008).

Volatile compounds analysis

Solid-phase microextraction (SPME) and GC–MS analysis were performed following the method described by Corral, Salvador, and Flores (2013) using a 85 μm Carboxen/Polydimethylsiloxane (CAR/ PDMS) fiber (Supelco, Bellefonte, PA) installed in a Gerstel MPS2 multipurpose sampler (Gerstel, Germany) and an Agilent HP 7890 series II GC with an HP 5975C mass selective detector (Hewlett-Packard Palo Alto, CA, USA). The volatile compounds (VOCs) detected were identified by comparison with mass-spectra from the library database (Nist'05), linear retention index (van Den Dool and Dec. Kratz, 1963) and by comparison with authentic standards. The quantification of volatile compounds was done in SCAN mode using either total or extracted ion chromatogram (TIC or EIC) on an arbitrary scale.

A gas chromatograph (Agilent 6890, USA) equipped with an FID detector and sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) was used to analyze aroma compounds extracted by SPME as described by Corral *et al*. (2013). The detection frequency (DF) method was used to estimate the aromatic impact of each volatile and each assessment was carried out according to Olivares, Navarro, and Flores (2011). Four trained panelists evaluated the odors from the GC-effluent. Each assessor evaluated 3 sausages for a total of 12 assessments, the final DF was obtained by summing the 12 sniffings. The detection of an odor by less than three assessors was considered noise. Compounds were identified by comparison with mass spectra, with linear retention indices of authentic standards injected in the GC–MS and GC-O, and by coincidence of the assessors' descriptors with those reported by Burdock (2010).

Sensory analysis

Table 7. Analysis on burgers and dry-fermented sausages

		Burgers	**Dry-fermented sausages**
pH		X	Crison GLP21
a_w		Lab Master-aw (NOVASINA AG)	ISO 21807:2004
Color (L*, a*, b*)		Minolta CR-300	Minolta CR-200
Moisture		AOAC, 2000: 935.29	Martillotti *et al.*, 1987
Ether extract		X	Martillotti *et al.*, 1987
Fat content		(Pérez-Palacios *et al.*, 2008)	X
Protein		AOAC, 2000: 992.15	AOAC, 2012: 976.05
Ash		X	AOAC, 2012: 920.153
Fatty acids profile		Lipid extraction: (Folch, Lees and Sloane Stanley, 1957) Fatty acid methyl esters: AOAC, 2000: 963.22	Lipid extraction: (Folch, Lees and Sloane Stanley, 1957) Fatty acid methyl esters: (Pugliese *et al.*, 2009)
Aromatic profile		X	SPME-GC-MS (Corral, Salvador and Flores, 2013)
Lipid oxidation (TBARs)		(Salih *et al.*, 1987)	(Vyncke, 1970)
Texture analysis (TPA)		X	Zwick-Roll Z2.5 (loading cell 1kN, speed 1 mm/s)
Sensorial analysis	**Quantitative-descriptive**	(Jiménez-Martín *et al.*, 2015)	(Pugliese *et al.*, 2010)
	Olfactometry	X	GC-O (Olivares, Navarro and Flores, 2011; Corral, Salvador and Flores, 2013)

Sensory analysis was carried out in an equipped laboratory by 8 trained panelists using a quantitative-descriptive analysis method. Fourteen attributes (grease appearance, abnormal colors, firmness, color uniformity, redness, cured meat flavor, off odor, salty, rancid, off flavor, hardiness, juiciness, aftertaste, general acceptability) were evaluated, each attribute was scored in a 10 cm non-structured line (Pugliese *et al*., 2010). Select subjects underwent an introductory session, where the testing procedures and the chosen sensory traits were discussed using two types of comparable commercial products. During three sessions, panelists evaluated a total of 9 sausages (3 samples × 3 treatments) identified by an alphanumerical code. The sausages were divided in 0.5 cm-thick × 2 cm-diameter slices and two slices of each samples were randomly served to judges at room temperature (20 °C). Panelists were invited to eat a cracker and drink a glass of water between samples.

4. Statistics

4.1. Burgers

The effect of cooking and type of enrichment were analyzed by two-way ANOVA using SAS. (1996) SAS/STAT software, release 9.4. When significant differences were observed ($p < 0.05$), they were evaluated by a Tukey's test. The following model was used:

$Yijk = \mu + Ti + Aj + eijk$

Where: Y is the j^{th} observation, μ is the overall mean, T is the i^{th} treatment; A is the j^{th} addition and e_{ij} is the error, which is an independent random variable. The interaction between factors was tested, but it resulted not significant for any variable.

For the sensorial data the following model was used:

$Yijkl = \mu + Ai + Pj + Tk + eijkl$

Where: Y is the j^{th} observation, μ is the overall mean, A is the i^{th} addition; P is the j^{th} panelist, T is the k^{th} treatment and e_{ijk} is the error, which is an independent random variable.

4.2. Dry-fermented sausages

Data were analyzed by SAS software. Two-way ANOVAs were performed on physical and chemical data according to the following model:

$Yijk = \mu + Ti + Bj + \varepsilon ijk.$

Where μ is the mean, T is the i^{th} treatment, B is the j^{th} batch and ε is the error. For sensory data, effect of panelist was included in the previous model. The interaction between Treatment and Batch factors was tested but being not significant, it was not included in the model.

Volatile compounds data were also analyzed by a multivariate approach to determine the presence of characteristic compounds able to be allocated to samples among different treatments. A stepwise discriminant analysis (SDA) was first used to reduce the space-variables, selecting the subset of variables that better discriminated groups. Canonical discriminant analysis (CDA) were performed using SDA

selected variables, resulting in 2 new variables, called canonical functions (CAN1, CAN2). They consisted of a series of canonical coefficients (CC) that indicate the partial contribution of each variable in composing the CANs. The greater the CC, the more the variable contributes to CAN composition.

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RESULTS

Chapter 1

Effects of different protein levels on nitrogen balance, in vivo performances and slaughtering traits of Cinta Senese growing pigs

Submitted to Journal of feed science and technology:

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Effects of different protein levels on nitrogen balance, in vivo performances and slaughtering traits of Cinta Senese growing pigs

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Abstract: Four different crude protein levels (120, 140, 160 and 180 g/kg as fed basis, named CP12, CP14, CP16 and CP18, respectively) were tested to assess the optimal protein requirements of Cinta Senese during the growing phase. *In vivo* performances, slaughtering traits and nitrogen balance were evaluated using individual pens and metabolic cages. Results on *in vivo* performances showed a negative impact of increasing crude protein levels on feed intake, average daily gain and protein conversion. Carcass composition and tissues percentage were not affected by dietary treatment but, differences between low (CP12+CP14) and high (CP16+CP18) protein diets were observed for fat and lean gain, as well as for protein conversion in lean and in lean protein. The nitrogen balance corroborated these results showing a significantly higher loss of N through urine as the dietary CP levels increased and, consequently, a worsening in the biological value of the HP diets. The overall results indicated that, among the tested diets, the CP12 diet was adequate to fulfil Cinta Senese protein requirements during the growing phase.

Key words: Swine nutrition; Nitrogen emissions; Local breed; Carcass quality

Highlights:

- Cinta Senese protein requirements in growing were fulfilled by a 120g/kg CP diet
- Different protein levels had no effects on carcass quality traits
- Diets providing 160 and 180g/kg of CP resulted in higher N excretions
- Increasing protein levels worsened the nutritional value of the diet

1. Introduction

Cinta Senese is a Tuscan native pig breed, traditionally reared outdoor in extensive systems. It shares with most of the local pig breeds many physiological traits typical of the obese genotypes as slower growth rate and a higher predisposition to fat deposition than commercial pig breeds (Lebret, 2008). In the past years, the assessment of the optimal protein intake and protein:energy ratio for growing pigs was of central importance for pig nutrition research, but it was limited to selected genotypes (Wecke and Liebert, 2009; Whittemore, 1983). Taking into account the specific metabolic features of native breeds, proper feeding strategies and ad hoc diet formulations are also needed to fulfil their nutritional requirements and enhance their performances (Bonneau and Lebret, 2010). Earlier studies on Cinta Senese pigs from 50 to 145 kg (Sirtori et al., 2010; Sirtori et al., 2014) and on Iberian pigs from 15 to 50 kg (Nieto et al., 2002) and from 50 to 100 kg of body weight (Barea et al., 2007), agreed that the protein requirements of these breeds are lower than the selected genotypes ones. Similarly, Nieto et al. (2014), in a meta-analysis, observed how the Iberian pigs follow a markedly different pattern of relative growth of carcass components compared to lean and conventional genotypes, confirming the need to better understand the nutritional requirements of local pig breeds for each stage of growth. The use of feeding models based on selected genotypes' performances either for native breeds feeding, might lead to an excess in dietary protein that the latter breeds cannot use for protein accretion and expulse through urine. This represents an energetic cost for the animals and an economic loss for farmers. Nevertheless, besides the economic convenience, defining the optimal balance between protein and energy intakes also for local pig breeds would have positive consequences on the environment, lowering the nitrogenous excretions of pig farms (van Milgen and Dourmad, 2015; Wang Y. et al., 2018). This is particularly important for

local breeds that are traditionally reared outdoor, and they are more exposed to the risk of nitrogen leaching (Eriksen et al., 2006; Halberg et al., 2010; Jørgensen et al., 2018).

To better understand Cinta Senese protein requirements during the growing phase, from 30 to 80 kg of live weight, the effect of protein level on *in vivo* performances, slaughtering traits and nitrogen balance was studied.

2. Materials and methods

The trials were carried out in the facilities of the Department of Agriculture, Food and Environmental Science -DISPAA – Florence, according to the EU Directive 2010/63/EU for animal experiments (experimental protocol approved by Authorization n 84/2016-PR – 28/01/2016).

2.1. Diets

The tested diets contained four protein levels: 120, 140, 160 and 180 g/kg as fed basis (named CP12, CP14, CP16 and CP18, respectively) and had the same protein quality in terms of balance in essential aminoacids. Diets formulation was made according the Nutrient Requirements of Swine (NRC, 2012), considering, as reference, protein and AAs requirements of pig of 50 kg (16% of CP as feed) and modifying the other diets taking into account the AAs balance, according the ideal protein composition. Table 1 shows ingredients and chemical composition for each dietary formulation. Diets were pelleted to avoid single ingredients to be selected by the animals. Animals were fed twice a day (at 8.00 am and 4.00 pm in equal meals). Two percent of bentonite was added to each formulation to increase the Acid Insoluble Ash (AIA), used as internal marker to assess diet's digestibility.

Table 1 - Ingredients and chemical composition of diets (% on d.m.)

		Diet			
		CP12	**CP14**	**CP16**	**CP18**
Ingredients					
Maize		73.50	68.00	62.95	57.40
Soybean meal (46%)	%	9.00	14.50	19.50	25.00
Wheat bran	"	10.00	10.00	10.00	10.00

Maize oil	“	2.00	2.00	2.00	2.00
Bentonite	“	2.00	2.00	2.00	2.00
Lysine HCl		0.45	0.45	0.45	0.50
Methionine		0.05	0.05	0.10	0.10
Premix[1]	“	3.00	3.00	3.00	3.00
Composition					
Dry matter	%	87.92	88.18	88.16	88.07
Crude Protein	“	13.34	15.58	17.86	20.37
Ether extract	“	4.87	4.71	4.73	4.76
NDF		19.91	18.89	19.38	17.69
ADF		6.60	7.37	7.63	8.61
ADL		1.46	1.79	2.11	1.99
Ash	“	6.81	7.58	7.69	7.68
Lysine[2]	“	0.99	1.15	1.30	1.46
Methionine[2]		0.31	0.34	0.42	0.44
Gross energy[3]	MJ/kg	18.296	18.270	18.402	18.570

[1] Premix provided per kg of feed: Vitamin A: 13500 IU; Vitamin D3: 1200 IU; Vitamin E: 12 mg; Vitamin K: 2.25 mg; Vitamin B1: 2.4 mg; Vitamin B2: 4.8 mg; Vitamin B6: 1.8 mg; Vitamin B12: 0.024 mg; Niacinamide 24 mg; Calcium pantothenate: 1.35 mg; Folic acid: 0.3 mg; Choline HCl 495 mg; Iron(II) carbonate 90 mg; Copper(II) sulfate 93 mg; Manganese oxide 30 mg; KI 1.96 mg; Na Selenite 0.3 mg; DL Methionine 105 mg; L Lysine 60 mg.

[2] Calculated from tabulated data of the ingredients

[3] Calculated from specific values of the analytical components

2.2. *In vivo* performances and slaughtering traits

Twelve Cinta Senese castrated males, of 28 kg of live weight and 135-days old on average, were divided in 4 dietary groups and allocated in individual pens provided with automatic nipple for water and chains according to animal welfare requirements. Individual boxes were divided in three blocks of 4 boxes in the barn were the trial took place and inside each block every dietary treatment was tested. The trial was repeated twice for a total of 24 pigs and 6 animals for each diet. The feeding level adopted was 0.95 x *ad libitum* and troughs were refilled thrice a day. Feed was stored separately for

each individual pen in order to weekly calculate the feed consumption by weight's difference between one week and the following one. The first and the second replications lasted 6 and 7 weeks respectively, when animals reached the average target weight of 65 kg and they were slaughtered together to determine the carcass composition, tissues distribution and nitrogen content. Six more pigs were slaughtered at the beginning of the first trial, at the average weight of 28.8 kg; in line with the initial live weights of the 24 pigs in trial. They were used to estimate the initial carcass composition and nitrogen content of the pigs in trial, according to the comparative slaughter method. After slaughtering, the two half-carcasses of each animal were weighed separately, cooled for 12 hours and then the right side was dissected in the anatomical cuts: head, neck, shoulder, ribs, loin plus belly, and ham, all comprehensive of the surrounding subcutaneous fat and skin. The main cuts were weighed and then divided in subcutaneous fat plus skin, intermuscular fat, lean and bone. Tissues were weighed separately and sampled to be analyzed, whereas bone was discarded. Subcutaneous fat, intermuscular fat and lean (*Longissimus dors*i (LD) separately) of each cut were analyzed for proximate composition according the official methods (AOAC, 2012). Total lean, fat and bone of carcass were calculated and the total nitrogen content in lean was also obtained as sum of the individual cuts' composition. Moreover, the gain in lean, fat, bone and nitrogen, during the whole trial was calculated as difference between the final and the initial composition, estimating the latter by the regression on live weight and anatomical cuts of the six piglets slaughtered at beginning of the trial.

2.3. Digestibility and nitrogen balance

Eight Cinta Senese castrated males were divided in two groups of 4 pigs each and they underwent 8 experimental cycles in metabolic cages (60 x 130 cm). Every animal tested all the diets at different ages, according a Latin square design. The trial lasted a total of 9 weeks, animals were 5 months old, they weighed on average 55 kg at the beginning of the trial and 75 kg at the end. The same four formulations previously described in Table 1 were contemporarily tested by four alternating pigs that occupied the four available cages. Animals were fed twice a day (at 8.00 am and 4.00 pm in equal meals). Daily feed allowance was 90 g/kg of metabolic weight. When present, refusal was collected before the morning distribution to calculate the actual intake in the previous day. Cages were

supplied with water nipple and trough and were adapted to separately sampling faeces in trays and total urine in flasks containing 20 ml of 8N sulphuric acid to avoid ammonia loss (Sardi et al., 1998). Each cage period lasted 5 days and was preceded by a 9-day period on floor for adaptation to diet. The cage period consisted of a phase of adaptation (2 days) and a phase of faeces sampling and total urine collection (3 days) according to Zhang and Adeola (2017) and in line with other total tract digestibility trials (Nieto et al., 2002; Acciaioli et al., 2011; Wang et al., 2017); the length of cage period was planned to address the ministerial prescription on animal welfare (Authorization n 84/2016-PR – 28/01/2016). Room temperature was set at 21°C and relative humidity was about 80%. Animals were weighed before each turn in cage to adjust the amount of feeding on their metabolic weight. The collection of total urine and faeces samples took place at fixed hours, twice a day for faeces (9.00 am and 4.00 pm) and once a day for urine (4.00 pm). The daily urine production of each subject was weighed and then a sample of the total urine was taken. Faeces and urine collected in a day were associated to the feed intake of the previous day to calculate digestibility and nitrogen balance.

2.4. Chemical analysis

The analytical methods used for feed, faeces and urines are the follows: moisture (AOAC, 2012, ref: 934.01), crude protein (AOAC, 2012, ref: 976.05), ether extract (AOAC, 2012, ref: 950.46), ash (AOAC, 2012, ref: 942.05), NDF (Goering, 1970), ADF and Lignin (AOAC, 2012, ref: 973.18. Dietary formulations and faeces were also analyzed for acid-insoluble ash (AIA). AIA was used as undigestible internal marker to calculate the total tract apparent digestibility (TTAD) of dietary components following the method proposed by Van Keulen and Young (1977). Similarly, on meat and fat samples, chemical analysis to determine moisture (AOAC, 2012, ref: 950.46), protein content (AOAC, 2012, ref: 976.05), ash (AOAC, 2012, ref: 920.153) and ether extract (AOAC, 2012, ref: 991.36) were carried out.

2.5. Statistical analysis

Data were analyzed by GLM Procedure (SAS, 2007) using the following model:

2.5.1. In vivo performances and slaughtering traits

$Y_{ijkl} = \mu + P_i + T_j + B_k + b*X_{ijkl} + E_{ijkl}$

Where

$Y = l^{th}$ observation;

$P = i^{th}$ Protein content (1, .. 4)

$T = j^{th}$ Trial (1, 2);

$B = k^{th}$ Block of pens (1,.. 3).

X = continuous effect of Initial live weight of pig;

E = random error.

2.5.2. Digestibility and nitrogen balance

$Y_{ijkl} = \mu + P_i + S_j + D_k + c*X_{ijk} + E_{ijkl}$

Where

$Y = l^{th}$ observation on j^{th} subject;

$P = i^{th}$ Protein content (1, .. 4)

$S = j^{th}$ Subject;

$D = i^{th}$ Day of sampling (1, ..3)

X = continuous effect of Metabolic weight of pig at entry in cage;

E = random error.

Statistical differences among mean values were assessed using Student's t test, with the level of significance established at 5%. The dietary formulations were clustered according their CP levels in low protein (LP) formulations (CP12+CP14) and high protein (HP) formulations (CP16+CP18), considering as medium point the CP supplementation usually adopted for selected pig breeds. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of dietary treatment and the significance of difference between LP and HP groups.

3. Results

3.1. *In vivo* performances and slaughtering traits

The dietary CP level supplied affected *in vivo* performances of Cinta Senese growing pigs. Most of the examined traits showed a significant linear response pattern to increasing CP levels in diet, whereas quadratic regression was never significant, resulting also in a clear differentiation between LP and HP formulations (Table 2). Diet significantly affected the final weight (P=0.050), even if the animals were chosen of the same live weight. Animals fed the CP12 treatment resulted heavier than CP16 and CP18 animals, whereas CP14 animals were comparable to CP12 and CP16 ones. Also, total

feed intake (P=0.045) and protein conversion index (P<0.0001) were similarly affected by diet, with CP12 animals showing the total feed intake higher than CP18 ones and the lowest Protein conversion index (PCI). Contrariwise, feed conversion index (FCI) was not affected by dietary treatment (P=0.334). At the end of the trial the LP and HP animals resulted significantly different for all the examined traits, except, again, for FCI (P=0.096). At increasing levels of dietary CP corresponded a sharply marked linear decrease of total feed intake (P=0.006). The decrease of ADG was close to linearity (P=0.014), specifically, the ADG of HP animals was lower than the LP ones (P=0.009). Therefore, the PCI resulted markedly lower for LP than for HP formulations (P<0.0001). Carcass yield and composition (Table 3), did not vary among the four dietary groups, except for a linear increasing trend for ham percentage (P=0.020); however, it is worth noting the behavior of kidneys' percentage on carcass that followed a linear increase from CP12 to CP18 (P=0.011) with a clear differentiation between LP and HP animals (P=0.013).

No significant differences among dietary groups were observed for tissues composition, as well as for lean, fat and bone gain (P=0.120, P=0.080 and P=0.344 respectively) (Table 4). Lean and fat daily gains followed a linear decreasing trend (P=0.048 and P=0.012, respectively). Similarly, the conversion rate of dietary protein in lean an in lean protein linearly decreased from CP12 to CP18 and from HP to LP diets (P<0.0001), being also noticeably lower for LP animals (P=<0.0001), whereas the feed conversion in lean was not affected by protein level (P=0.397), agreeing with *in vivo* results.

Table 2. Effects of different dietary levels of ideal CP on *in vivo* performances of Cinta Senese growing pigs.

	Diet				RMSE	P			
	CP12	**CP14**	**CP16**	**CP18**		**Diet**	**Linear**	**Quadratic**	**LP[1] vs HP[2]**
Initial weight (kg)	28.26	27.57	28.56	28.36	2.73	-	0.675	0.589	-
Final weight (kg)	62.96 a	62.61 ab	60.55 b	60.38 b	1.81	0.050	0.010	0.905	0.008
Total feed intake (kg)	113.11 a	112.12 a	110.57 ab	108.10 b	2.83	0.045	0.006	0.545	0.014
Total CP intake (kg)	12.99 d	15.16 c	17.06 b	18.93 a	0.42	<0.0001	0.014	0.411	<0.0001
ADG (kg/d)	0.76	0.75	0.71	0.71	0.04	0.064	0.014	0.914	0.009
FCI	3.25	3.27	3.43	3.47	0.18	0.334	0.165	0.584	0.096
PCI	0.37 d	0.44 c	0.53 b	0.59 a	0.03	<0.0001	<0.0001	0.704	<0.0001

Abbreviations: ADG, average daily gain; FCI, feed conversion index, (kg feed/kg weight gain); PCI, protein conversion index, (kg of protein intake/kg weight gain)

[1] Low protein diets: CP12 + CP14

[2] High protein diets: CP16 + CP18

Different letters in the same row indicate significant differences ($p<0.05$) among diets (a, b, c, d)

Table 3. Effects of different dietary levels of ideal CP on carcass composition and tissues of Cinta Senese growing pigs.

	Diet					P			
	CP12	**CP14**	**CP16**	**CP18**	**RMSE**	**Diet**	**Linear**	**Quadratic**	**LP[1] vs HP[2]**
Carcass weight (kg)	52.49 a	51.57 a	49.40 b	48.83 b	1.67	0.005	0.001	0.810	0.001
Carcass yield (kg)	83.35	82.35	81.60	80.86	2.24	0.304	0.067	0.895	0.105
Carcass composition (%)									
Head	8.93	8.26	8.94	9.24	0.69	0.174	0.235	0.118	0.112
Neck	8.99	8.75	8.64	8.85	0.74	0.865	0.711	0.472	0.703
Shoulder	13.67	13.69	13.32	13.71	0.56	0.599	0.803	0.446	0.492
Ribs	25.69	26.16	24.98	25.35	1.25	0.455	0.360	0.928	0.168
Loin and belly	14.91	15.14	15.53	14.54	0.87	0.305	0.657	0.116	0.986
Ham	25.26	25.56	25.98	26.16	0.64	0.116	0.020	0.789	0.029
Kidney	0.35 b	0.40 ab	0.46 a	0.46 a	0.08	0.050	0.011	0.497	0.013
Tissues (%)									
Total lean	38.28	38.28	37.16	39.02	1.68	0.813	0.509	0.549	0.664
Total fat	45.38	45.20	45.38	43.72	2.64	0.669	0.346	0.513	0.518

Table 3. Effects of different dietary levels of ideal CP on carcass composition and tissues of Cinta Senese growing pigs.

	Diet					P			
	CP12	**CP14**	**CP16**	**CP18**	**RMSE**	**Diet**	**Linear**	**Quadratic**	**LP[1] vs HP[2]**
Subcutaneous fat	36.24	36.52	36.35	34.94	2.42	0.680	0.372	0.413	0.475
Intermuscular fat	6.55	6.28	6.37	6.55	0.60	0.839	0.926	0.386	0.852
Total bone	14.71	14.93	14.52	15.26	1.19	0.744	0.579	0.624	0.883

[1] Low protein diets: CP12 + CP14

[2] High protein diets: CP16 + CP18

Different letters in the same row indicate significant differences ($p<0.05$) among diet (a, b, c, d)

Table 4. Effects of different dietary levels of ideal CP on slaughtering traits and protein deposition of Cinta Senese growing pigs.

	Diet				RMSE	P			
	CP12	**CP14**	**CP16**	**CP18**		**Diet**	**Linear**	**Quadratic**	**LP[1] vs HP[2]**
Daily gain (g/d)									
Lean	272.95	268.63	245.43	252.98	20.79	0.120	0.048	0.507	0.025
Fat	357.83	346.82	329.85	307.0	32.09	0.080	0.012	0.668	0.023
Bone	82.54	82.77	70.42	77.94	12.85	0.344	0.291	0.510	0.137
Protein of lean (g/d)	59.40	58.99	53.89	54.95	5.78	0.295	0.107	0.767	0.067
Conversion rates (kg/kg)									
Feed conversion in lean	9.13	9.38	9.99	9.52	0.86	0.397	0.286	0.334	0.188
Protein conversion in lean	1.05 c	1.27 b	1.54 a	1.66 a	0.13	<0.0001	<0.0001	0.366	<0.0001
Protein conversion in lean protein	4.82 c	5.80 b	7.00 a	7.65 a	0.69	<0.0001	<0.0001	0.579	<0.0001

[1] Low protein diets: CP12 + CP14

[2] High protein diets: CP16 + CP18

Different letters in the same row indicate significant differences ($p<0.05$) among diet (a, b, c, d)

3.2. Digestibility and nitrogen balance

Table 5 shows the feed intake and total tract apparent digestibility (TTAD) of the tested dietary formulations. The dry matter intake was the same for the 4 diets (P=0.153), with a slight negative decrease (P=0.034) from CP12 to CP18, already observed for the animals tested in the individual pens. The CP intake increased from CP12 to CP18 diets (P<0.0001), according the experimental design. The digestibility of the dietary components did not differ from one formulation to another, except for CP digestibility that resulted lower in CP12 than in the other diets. At increasing level of CP, the TTAD of dry matter followed a negative trend, outlined by the significance of linear regression (P=0.032), while the digestibility of CP increased (P=0.005). Dry matter, organic matter and ether extract digestibility scores were also significantly different (P=0.012, P=0.020 and P=0.034, respectively) comparing LP and HP groups. Table 6 shows the nitrogen balance of the four diets, all the examined parameters resulted affected by the dietary CP level. Nitrogen intake increased from CP12 to CP18 (P<0.0001) as well as faecal (P=0.001), adsorbed, urinary and total excreted N (P<0.0001), all showing the highest scores for pig fed the CP16 and CP18 diets. The above-mentioned parameters had a sharply clear positive trend (P<0.0001) from CP12 to CP18, and consequently, a marked separation between LP and HP diets was easily recognizable. It is worth noting that for urinary N, CP12 showed the lowest value even if compared to CP14. Retained N was lower for CP12 and CP16 diet than for CP14 and CP18 formulations, but did not follow a clear trend among diets, indeed nor linear (P=0.063) neither quadratic (P=0.222) trends were evinced. Eventually, the biological value (P=0.030) and the retained N/intake N ratio (P<0.0001) were more favorable for LP diets than for HP ones. Both the parameters linearly decreased from CP12 to CP18 formulations (P=0.001 and P=0.002), indicating a worsening in the nutritional value of the diets. Finally, even if the diets were formulated to be isoenergetic (see Table 1), providing the same amount of gross energy, the CP level influenced some energy parameters. Despite the increased faecal N, the digestible energy (DE) resulted unaffected by the diet formulation (P=0.267). Contrariwise, the metabolizable energy (ME) (P=0.028) and the ME:DE ratio (P=<0.0001) were higher for CP12 than for the other tested formulations, also outlining a significant linear decrease from CP12 to CP18 diets (P=0.044 and P<0.0001) and, for ME:DE, a clear differentiation between LP and HP groups (P<0.0001).

4. Discussion

Several studies have pointed out the physiological differences between selected and local-obese pig genotypes. The slower growth rate and lower predisposition to lean tissue deposition of the latter have been widely assessed for native pig breeds, suggesting the need of specific diet formulations for each phase of growth, either for these breeds (Liu et al., 2015; Nieto et al., 2012). Up to day, this knowledge is available only for Iberian pig, whose protein and energy requirements, as well as their optimal ratio, were studied along its whole life (Barea et al., 2007; Conde-Aguilera et al., 2011; Nieto et al., 2012, 2002). Similarly, also for Cinta Senese research started to investigate its protein requirements (Acciaioli et al., 2003; Sirtori et al., 2010; Sirtori et al., 2014) in order to adjust the diet formulations on its real protein requirements, helping to contain the relatively expansive cost of protein ingredient and to reduce nitrogen excretions in the environment. Indeed, adapting the dietary AA content to animal's different physiological needs according its life phase is a recognized strategy to lower N inputs and outputs while maintaining maximal performance (Millet et al., 2018). In the present work, all the tested diets provided the ideal protein, even if at increasing amount of dietary CP. The protein requirements are, essentially, AA requirements. If the dietary CP lowered, the fulfilment of the essential AA needs to be ensured. Hence, when feedstuff cannot provide enough of the essential AA, the reduction of the dietary CP is carried out with the addition of crystalline AA (Tuitoek and Lange, 1997; Soumeh et al., 2014; Wang Y. et al., 2018). Fail to meet the essential AA requirements, and their respective proportion, is one of the main reasons of N excretions. Thus, when the first-limiting AA is over, protein deposition stops and the AA in surplus were expulsed. Without limiting factors, protein deposition follows a linear response according to the amount of protein supplied. Once it reaches a breaking point, the protein deposition largely depends on energy supply (Nieto et al., 2002). The differences reported in this study on *in vivo* performances and slaughter traits, were neither due to unbalanced AA supplementation, nor to a shortage in energy supply, being the ME always higher than the recommended 15.70 MJ/kg dm for growing pigs (NRC, 2012).

Table 5. Feed intake and total tract apparent digestibility (TTAD) of components of the diets with different crude protein (CP) contents

	Diet				RMSE	P			
	CP12	**CP14**	**CP16**	**CP18**		**Diet**	**Linear**	**Quadratic**	**LP[2] vs HP[3]**
Intake (g/d)									
- Dry matter	1727	1709	1683	1598	196.00	0.153	0.034	0.422	0.070
- Crude protein	229 d	266 c	301 b	324 a	35.1	<0.0001	<0.0001	0.361	<0.0001
Total tract apparent digestibility									
- Dry matter	0.87	0.87	0.86	0.86	0.01	0.084	0.032	0.567	0.012
- Organic matter	0.90	0.90	0.89	0.89	0.02	0.124	0.061	0.989	0.020
- Crude protein	0.85 b	0.87 a	0.87 a	0.87 a	0.01	0.016	0.005	0.279	0.056
- Ether extract	0.93	0.93	0.92	0.92	0.03	0.197	0.081	0.952	0.034
- NDF	0.76	0.74	0.73	0.73	0.04	0.370	0.101	0.557	0.199

[1] Metabolic weight: (kg of live weight)$^{0.75}$

[2] Low protein diets: CP12 + CP14

[3] High protein diets: CP16 + CP18

Different letters in the same row indicate significant differences ($p<0.05$) among diet (a, b, c, d)

Table 6. Intake, balance and efficiency of utilization of Nitrogen of the diets with different crude protein (CP) contents

	Diet				RMSE	P			
	CP12	**CP14**	**CP16**	**CP18**		**Diet**	**Linear**	**Quadratic**	**LP[2] vs HP[3]**
N intake (g/d/kg MW)*	1.62 d	1.86 c	2.10 b	2.30 a	0.24	<0.0001	<0.0001	0.661	<0.0001
Nitrogen balance									
Fecal N **(**g/d/kg MW)	0.24 b	0.24 b	0.28 a	0.29 a	0.05	0.002	0.001	0.548	0.002
Absorbed N **(**g/d/kg MW)	1.38 b	1.62 b	1.82 a	2.00 a	0.21	<0.0001	<0.0001	0.512	<0.0001
Urinary N **(**g/d/kg MW)	0.67 c	0.81 b	1.13 a	1.11 a	0.14	<0.0001	<0.0001	0.010	<0.0001
Total excreted N **(**g/d/kg MW)	0.90 c	1.05 b	1.41 a	1.40 a	0.17	<0.0001	<0.0001	0.026	<0.0001
Retained N **(**g/d/kg MW)	0.72 b	0.81 a	0.70 b	0.90 a	0.23	0.018	0.063	0.222	0.523
Biological Value[4]	51.90 a	50.28 a	36.73 b	41.14 c	0.10	<0.0001	0.001	0.030	<0.0001
Retained/intake N	44.28 a	43.68 a	37.76 b	38.92 c	8.66	<0.0001	0.002	0.043	<0.0001
Digestible Energy (MJ/kg DM)	16.73	16.54	16.62	16.71	0.35	0.267	0.994	0.066	0.705
Metabolizable Energy (MJ/kg DM)	16.57 a	16.30 b	16.26 b	16.35 b	0.343	0.028	0.044	0.018	0.088
ME:DE	98.80 a	98.49 b	97.82 c	97.82 c	0.311	<0.0001	<0.0001	0.033	<0.0001

Abbreviations: ME, metabolizable energy; DE, digestible energy

[1] Metabolic weight: (kg of live weight)$^{0.75}$

[2] Low protein diets: CP12 + CP14

[3] High protein diets: CP16 + CP18

[4] Retained/Absorbed

[*] g/d per kg of Metabolic weight

Different letters in the same row indicate significant differences ($p<0.05$) among diet (a, b, c, d)

The earlier studies on Cinta Senese (Acciaioli et al., 2003; Sirtori et al., 2010; Sirtori et al., 2014) concerned the whole growing-fattening period, highlighted its lower protein requirements compared to selected genotypes, whose recommended protein intake for growing pigs is 16% of CP (NRC, 2012). In these studies, the authors indicated the 10% of CP as the optimal dietary protein level for growing-finishing period (from 46 to 150 kg), partially agreeing with our results, also considering that the present study refers to the growing phase alone (from 28 to 65 kg). In growing pigs, the protein requirements are higher than during finishing period, being mainly related to protein deposition processes and, in a lesser part, to maintenance (van Milgen and Noblet, 2003). The overall results of *in vivo* performances and slaughtering traits suggest that supplying increasing levels of dietary CP to Cinta Senese growing pigs did not have positive effects on performances (ADG, PCI, lean gain, protein conversion in lean and in lean protein); contrariwise, from CP12 to CP18, the efficiency in protein utilization and N retention worsened. In Iberian pigs from 15 to 50 kg of lw Nieto et al. (2002) observed that the dietary CP content affected ADG, protein deposition and retained/intake N ratio. Near the *ad libitum* feeding level, the best performances for these parameters were associated to a dietary CP content of 129 g/kg of dry matter, comparable to our CP12 formulation, while increasing content of dietary CP gradually lowered the performances. These results corroborated the evidences on the metabolic differences between selected and obese genotypes, thus, Cinta Senese pigs were able to adsorb high amount of N at ileal level. It can be assumed that they have an adequate enzymatic profile to degrade dietary formulation up to CP18, but once AA are adsorbed and the genetic upper limit for protein deposition is reached, AA are used in catabolic processes. The poor efficiency of protein catabolism is widely known (van Milgen and Noblet, 2003; Wang Y. et al., 2018; Whittemore, 1983), and the negative effects of this pathway affects overall animal performances. In lean genotypes, genetic selection has raised the predisposition to depot lean tissue beyond the upper limit of appetite, making high-protein diets fully exploitable by animals (Nieto et al., 2002). The effects of protein on food intake has been fully studied and it is mainly attributable to hormonal satiety responses involving cholecystokinin as well as glucagon-like peptide-1, peptide tyrosine and gastric inhibitory polypeptide (Psichas et al., 2015; Roura and Fu, 2017; Wang C., 2018). Especially, the response of cholecystokinin has been found to be different between slow-

growing and fast-growing pigs, with the formers reaching earlier a higher concentration of cholecystokinin in plasma, after the feed ingestion started (Tauson, 2003). Besides the appetite depression evinced both for individual penned animals and those in metabolic cages, the greater CP content of the HP diets, have ensured a higher intake of CP, so the worse *in vivo* performances and the lower lean and protein accretion indices observed for HP pigs, were mainly related to a poorer protein utilization efficiency. Additionally, the greater weight of kidneys as the dietary CP increased, suggests a higher metabolic activity in expulsing ureic N, as reported also for Iberian pigs fed increasing level of dietary CP (Barea et al., 2007; Nieto et al., 2015). These results corroborated the nitrogen balance, clearly delineating a positive response of urinary N, that increased according to the dietary CP supplied. It can be concluded that the LP diets were adequate to fulfil Cinta Senese protein requirements during growing, indeed providing a CP content equal or greater than the recommended for high-performance breeds, had no effect on carcass composition and tissues percentages, protein and lean gain were not enhanced but depressed by increasing levels of dietary CP and N excretions linearly increased. Moreover, considering the LP diets, most of the examined parameters were not influenced except for lean and fat gain and protein conversion in lean protein, that were significantly better for CP12 animals than for CP14 ones. So, the CP12 diet seems the more appropriate for this growth stage. It is worth noting that this study failed in identifying a minimum level of dietary CP below which growth performances are affected by the CP's shortage. Results observed by Sirtori et al. (2010) discouraged the use of diets providing less than 10% of CP even in the fattening phase, for this reason, in the present research, the minimum was stated at CP12. The opportunity of further lowering of the dietary CP for Cinta Senese growing pigs cannot be excluded, but the greater fat gain observed in LP animals might indicate that we reached the lower limit of protein to energy supply below which, lacking the AA for protein accretion, the energy in excess is used to fat deposition. Indeed, diets excessively poor in protein, enhance the genetic predisposition to fat deposition of local breeds, leading to carcasses extremely fat (Wang Y. et al., 2018) with a worse slaughtering yield and a lower consumer acceptability of meat.

Conclusions

Among the tested dietary formulations, the CP12, providing 120g/kg of crude protein to growing Cinta Senese pigs, resulted adequate to fulfill their protein requirements. The CP12 formulation had not negative effects on *in vivo* performances and quality carcass traits, whereas resulted in an amelioration of the nitrogen balance, lowering the nitrogen excreted through urine.

Acknowledgments

The research was carried out with funds from the European Union's Horizon 2020 research and innovation program under grant agreement No 634476 (acronym TREASURE). The content of this works reflects only the author's view and the European Union Agency is not responsible for any use that may be made of the information it contains.

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Chapter 2

Enrichment of Cinta Senese burgers with omega-3 fatty acids. Effect of type of addition and storage conditions on quality characteristics

Published on GRASAS Y ACEITES as:

Aquilani, C., *et al.* (2018) 'Enrichment of Cinta Senese burgers with omega-3 fatty acids. Effect of type of addition and storage conditions on quality characteristics', *Grasas y Aceites*, 69(1), e235. doi: 10.3989/gya.0671171.

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Personal contribution: Physical, chemical and sensorial analysis, statistical elaboration, writing and editing of the paper as corresponding author.

GRASAS Y ACEITES 69 (1)
January-March, 2018, e235
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0671171

Enrichment of Cinta Senese burgers with omega-3 fatty acids. Effect of type of addition and storage conditions on quality characteristics

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Submitted: 26 June 2017; Accepted: 30 October 2017

SUMMARY: The most beneficial omega-3 PUFAs to human health, EPA and DHA fatty acids, are typically present in fish products, but extraneous to meat. Therefore, Cinta Senese pork burgers were added with microencapsulated (M) and bulk fish oil (F) and subjected to three storage conditions: no storage (T0), chilled (T5) and frozen storage (T30). The physico-chemical and sensory attributes of raw and cooked burgers were investigated. After storage and cooking, EPA and DHA were better preserved in M burgers than in F samples, which showed the highest TBAR values at T0 and T5, while M samples presented scores similar to the control. Panelists observed differences mainly in greasy appearance, odor intensity and cooked meat odor and flavor. The M group showed the best scores at T5 with respect to the control and F burgers. So, fish oil microencapsulation was an effective method to prevent EPA and DHA oxidation while respecting burger quality characteristics.

KEY WORDS: *Fish oil; Meat quality; Microencapsulation; Pork; Sensorial attributes*

RESUMEN: ***Enriquecimiento de la hamburguesa Cinta Senese con ácidos grasos omega-3. Efecto del tipo de adición y condición de almacenamiento en las características de calidad.*** EPA y DHA son los ácidos grasos poliinsaturados omega 3 más beneficiosos para la salud humana, se presentan típicamente en el pescado, y no se encuentran en carnes. Por ello, se elaboraron hamburguesas de cerdo de la especie "Cinta Senese" añadiendo aceite de pescado (F), microcápsulas que contenían aceite de pescado (M) o sólo a base de carne (control (C)) y se mantuvieron bajo las siguientes condiciones de almacenamiento: sin almacenaje (T0), en refrigeración (T5) y congelación (T30). Se estudiaron los atributos sensoriales y físico-químicos de las hamburguesas crudas y cocinadas. En cuanto al almacenamiento y el cocinado, las hamburguesas con microcápsulas preservaron mejor los EPA y DHA que las muestras con aceite de pescado, las cuales presentaron los valores más altos de TBARs en las muestras T0 y T5, mientras que las M mantuvieron unos resultados similares a las de tipo control. En los resultados de la cata realizada se observaron, entre los tratamientos realizados y respecto a las distintas condiciones de almacenamiento, diferencias en la apariencia grasienta, olor y flavor a carne cocida e intensidad de olor. En las hamburguesas M se obtuvieron las mejores puntuaciones frente a las encontradas en F y C en el tipo T5. Por tanto, la microencapsulación de aceite de pescado se verificó como un método efectivo para prevenir la oxidación de EPA y DHA respetando la calidad y características de las hamburguesas.

PALABRAS CLAVE: *Aceite de pescado; Calidad de la carne; Características organolépticas; Cerdo; Microencapsulación*

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Citation/Cómo citar este artículo: Aquilani C, Pérez-Palacios T, Sirtori F, Jiménez-Martín E, Antequera T, Franci O, Bozzi R, Acciaioli A, Pugliese C. 2108. Enrichment of Cinta Senese burger with omega-3 fatty acids. Effect of type of addition and storage condition on quality characteristics. *Grasas Aceites* **69** (1), e235. https://doi.org/10.3989/gya.0671171

1. INTRODUCTION

Alpha-linolenic acid (ALA C18:3 n-3) and its longer-chain metabolites, i.e. eicosapentaenoic acid (EPA C20:5 n-3) and docosahexaenoic acid (DHA C22:6 n-3), are important polyunsaturated fatty acids (PUFAs), due to their role in the prevention and treatment of cardiovascular diseases, some types of cancer and immune inflammatory diseases (Garcia-Almeida *et al.,* 2010; Pelliccia *et al.,* 2013; Dienke *et al.,* 2016). However, the real intake of these PUFAs in traditional Western diets is far below the recommended minimum of 250 mg per day of long-chain omega-3 PUFAs (Sanders, 2000). Indeed, the main sources of EPA and DHA are oily fish, oil fish supplements and to a lesser degree white fish and shellfish, which are hardly appreciated in most Western countries. Consequently, many studies focused on improving the nutraceutical quality of widespread foods by increasing their omega-3 content.

It is worth noting that the food selected to be enriched should be well accepted, popular, inexpensive and easy to cook. Moreover, it should constitute a potential opportunity to valorize products for the food industry, i.e. by adding value to less commonly accepted or traditional food. Diet habits are changing in accordance with a lifestyle that is focused on time-saving, and consequently, there is a growing demand for "ready-to-heat" products, such as burger meats. The popularity of these convenience products makes them a promising strategy to increase the intake of omega-3 fatty acids using enrichment methods.

Among animal products, meat and meat products appear to be an interesting target to be enriched because of their high consumption and fatty acid profile. Indeed, they are characterized by a low content of long-chain omega-3 PUFAs together with a high presence of monounsaturated fatty acids (approximately 45-50%) and saturated fatty acids (approximately 45–55%) (Givens *et al.,* 2006). Fortification can be carried out by feeding the animals with omega-3 enriched feedstuff (Corino *et al.*, 2014) or by enriching the products through a technological approach. In this last case, three main ways can be identified. The simplest one is directly adding fish (or vegetal) oil to food (Càceres *et al.*, 2008; Valencia *et al.*, 2008; Martínez *et al.*, 2012); another method is oil emulsification (Salminen *et al.*, 2013), which, in contrast to the former, provides PUFAs protection from lipid oxidation during the product's shelf-life, but it is unable to mask undesirable odors and flavors (mainly in the case of fish oil) (Jiménez-Colmenero,

2007). The last method consists of encapsulating the oil emulsion to form a single (or multi-) layer around each oil drop. This method is more complex, but it ensures the best results in preventing oxidation, preserving food sensorial attributes and avoiding the perception of fish or rancid flavors (Jiménez-Colmenero, 2007; Josquin *et al.*, 2012; Keenan *et al.*, 2015).

To meet consumer demand for healthier and more widely accepted meat products, burgers have been chosen for enrichment with fish oil as omega-3 source. The fortification could constitute an opportunity to re-valorize some products, such as Cinta Senese ones. This is a local pig breed reared extensively in Tuscany; its meat has obtained the Protected Designation of Origin (PDO) and has good perspectives for increasing its relevance. Its production is mainly focused on dry-cured products, above all hams and salami (Pugliese and Sirtori, 2012). Fresh meat from Cinta Senese pigs has not achieved a large diffusion in the market, which is likely related to the consumers' association of Cinta Senese only with dry-cured products and to the perception of fresh meat as unhealthy due to its high lipid content and low percentage of PUFA (Pugliese *et al.*, 2005). Therefore, the addition of omega 3 PUFAs to Cinta Senese fresh meat seemed to be an effective way to enlarge the market of Cinta Senese fresh products and to valorize this traditional breed.

The aim of the present study was to investigate the ease of producing omega-3 enriched burgers from Cinta Senese loins, given that their quality traits could be affected by the enrichment procedure (bulk fish oil vs. microencapsulated fish oil) and by the storage method typically used for burgers (chilled or frozen). The development of this type of product would improve the profitability of a local and high-quality pig production system.

2. MATERIALS AND METHODS

2.1. Burger manufacture and sampling

Burgers were made with loins of Cinta Senese pigs provided by Azienda Agricola Borgonovo (Cortona, AR, Italy). Ten pigs were bred outdoors and fed with commercial feed. Ten portions of fresh loins (a total of approximately 11kg of meat) were minced and mixed with salt (2%), sulfites (0.05%) and mashed potato powder (2.4%). Three types of burgers were made: control (C) (3.7 kg of mixture), with no further modifications, microcapsule burgers (M) adding 173 g of microcapsules to 3.7 kg of

mixture and fish oil burgers (F) adding 6 g fish oil to 3.7 kg of mixture. The respective quantities of microcapsules and fish oil were calculated to contain the same amount of EPA+DHA(1.67g).

The burgers were made by weighing 90g of mixture and shaping it into a standard burger mould.

For cooked samples, the following cooking procedure was used: grilling at 165 °C, flipping every 2 min until reaching an internal temperature of 73–75 °C, recorded using a thermometer probe (Testo 735-2, Lenzkirch, Germany).

Forty-eight burgers were made for each addition (C, M, F) and used as follows:

Five burgers underwent physico-chemical analysis as fresh matter; five burgers were cooked and used for physico-chemical analyses at 0 days (T0); seven burgers were cooked and underwent sensorial analysis at T0; five burgers were stored at 4 °C for 5 days (T5), then cooked and analyzed; seven burgers were stored under the same conditions (T5), cooked and examined by panelists; five burgers were stored at -20 °C for 30 days (T30), then cooked and analyzed; seven burgers were stored under the same conditions (T30), cooked and examined by panelists.

2.1.1. Physico-chemical analyses of raw and cooked burgers

The determinations carried out were: cooking loss, instrumental color and water activity; fat, protein, moisture content; fatty acid profile; lipid oxidation (TBARs).

A quantitative-descriptive sensorial analysis was carried out on cooked burgers only.

2.2. Fish oil

Fish oil was kindly provided by Biomega Natural Nutrients (Galicia, Spain). It is a low viscosity, vacuum deodorized oil. As reported by the producer, its omega-3 PUFA contents for 100 g of product are 5.96g of EPA and 25.83g of DHA.

2.3. Microcapsules

Multi-layer microcapsules were elaborated following the methodology of Jiménez-Martín *et al.*, (2016a) with some modifications. Fish oil (Biomega Natural Nutrients, Galicia, Spain) was used as a source of omega-3 PUFA (5.96% EPA, 25.83% DHA, 0.02% BHT). The process started from a primary emulsion made of 20 g of fish oil, 6 g of soy lecithin (Biogran S.L., Madrid, Spain) and dissolved in 174 g of water. This mixture was added to a solution made of 2 g of chitosan (Trades, Chitoclear FG 95,

Murcia, Spain) dissolved in 198 g of acetic acid (1%) (Scharlau, Barcelona, Spain) to form a secondary emulsion, which was homogenized (Homogenizer SPX, APV2000, Denmark) at 700 bar. This was added to 400 g of water and a maltodextrin (30%) (Roquette, Glucidex 12, Lestrem, French) solution for finally obtaining a feed emulsion. The feed emulsion (800g) was dehydrated and turned into powder using a laboratoryscale spray-dryer (Mini Spray Dryer B-290 Buchi, Switzerland) equipped with a 0.5 mm nozzle atomizer. The aspirator rate was adjusted to 80%, feed rate was 1 L/h, inlet temperature was 180 °C, and outlet temperature ranged 85–90 °C. The obtained microcapsules contained 9.63 mg of EPA+DHA per gram of microcapsules and 4% moisture, which was calculated following the methodology of JiménezMartín *et al.*, (2016b).

2.4. Physico-chemical analysis

Fresh (n=15) and cooked (n=45) burgers were first analyzed by means of instrumental color. Then, samples were minced, and water activity and moisture were immediately determined. The rest of sample was stored at -80 ºC until further analysis.

2.4.1. Cooking loss

Cooking loss was calculated as the difference between the weight of cooked and fresh burgers and it was expressed as a percentage.

2.4.2. Instrumental Color

Instrumental color was measured on the surface of the burgers. Fresh samples were examined the same day they were processed. In the case of cooked samples, measurements were taken when they had reached room temperature (20–25 °C). Instrumental color was determined using a Minolta CR-300 colorimeter (Minolta Camera Corp., Meter Division. Ramsey, NJ) with illuminant D65, a 0º standard observer and a 2.5 cm port/viewing area. The following color coordinates were determined: lightness (L^*), redness (a^*) and yellowness (b^*). The colorimeter was standardized before use with a white tile having the following values: L^*=93.5, a^*=1.0 and b^*=0.8.

2.4.3. Water activity

For the water activity, the system Lab Master-aw (NOVASINA AG, Switzerland) was used after calibration.

2.4.4. Moisture, fat content and protein

The moisture in fresh and cooked samples was determined gravimetrically at 100 ± 2 ºC by the official method (A.O.A.C., 2000a, reference 935.29).

Fat content was determined gravimetrically with chloroform:methanol (2:1, vol/vol), following the method described by Pérez-Palacios *et al.,* (2008).

Protein content was calculated in duplicate by the Kjeldahl method (A.O.A.C., 2000b, reference 992.15).

2.4.5. Fatty acids

Fatty acid methyl esters (FAMEs) from extracted fat were prepared by basic transesterification following the official method (A.O.A.C., 2000c, reference 963.22), using hexane and hydroxide potassium 2N. FAMEs were analyzed by gas-chromatography (GC) using a Hewlett–Packard HP-5890A gas chromatograph, equipped with an on-column injector and a flame ionization detector (FID), using a polyethylene glycol capillary column (Supelcowax-10, Supelco, Bellefonte, PA, USA) (60 m × 0.32 mm i.d. × 0.25 μm film thickness). The GC oven program temperature was as follows: initial temperature of 180 °C was raised at 5 °C/min to 200 °C, kept at this temperature for 40 min, raised at 5 °C/min to 250 °C, and then kept for an additional 21 min. The injector and detector temperatures were 250 °C. The carrier gas was helium at a flow rate of 0.8 ml/ min. Individual FAME peaks were identified by comparison of their retention times with those of standards (Sigma, St. Louis, MO, USA). Peak areas were measured and FAMEs were expressed as area percentage of total area FAMEs (%).

2.4.6. Lipid oxidation

Thiobarbituric acid-reactive substances (TBARS) were measured following the extraction method described by Salih *et al.,* (1987). Each burger was minced in a kitchen blender, and 2.5 g were homogenized for 2 min with 7.5 mL of 3.86% perchloric acid and 0.25 mL of butylated hydroxytoluene (4.2%). The tubes were kept on ice to avoid heat degradation. This homogenate was filtered and centrifuged (4 min, 3500 rpm). The supernatant (2 mL) was mixed with 2 mL of thiobarbituric acid 0.02 M. At the same time, a standard curve was prepared employing 1,1,3,3- tetraethoxypropane (TEP). Immediately, the mixture was heated to 90 ºC for 30 min, cooled and centrifuged again (2 min, 3500 rpm). Absorbance was measured at 532 nm on a spectrophotometer

(Hitachi U-2000, Tokyo, Japan). The concentration of malonaldehyde (MDA) was calculated from the standard curve, developed simultaneously with the samples using solutions of TEP (Merck, Schchardt, Germany). TBARS were expressed as mg MDA kg^{-1} sample.

2.5. Sensory analysis

All sessions were done in sensory panel rooms with the conditions specified in the UNE regulation (Norma UNE, 1979) equipped with white fluorescent lighting (220-230 V, 35 W). A piece of cooked burger (20g) was served hot on white plastic plates to panelists, marked with random three-digit codes. The panel sessions were held around 1-2 h before lunch time. Salt-free crackers and a glass of water at room temperature were provided to each panelist to rinse between samples. The burgers were assessed by a trained panel of 18 members using a descriptive analysis method. Eleven sensory traits grouped under appearance (greasy appearance), odor (odor intensity, cooked meat odor) texture (hardness, juiciness, oiliness), taste (salty) and flavor (cooked meat flavor, rancid flavor, flavor intensity, after taste) were assessed. Selected subjects underwent further training in meat and meat products sensory characteristics over five years. The number of burgers used for sensorial analysis was 7 for each supplementation, repeated for T0, T5 and T30. Each burger was divided into four parts to be served to panelists for a total of 28 pieces of burgers of each type (C, M, and F). Each panelist evaluated three pieces of burger in each session, and the sample order was randomized across assessors. Sensory traits were assessed by panelists in a 10 cm unstructured line, ranging from "less" to "more".

2.6. Statistical analysis

The effect of cooking and type of enrichment were analyzed by two-way ANOVA using SAS. (1996) SAS/STAT software, release 9.4. When significant differences were observed ($p < 0.05$), they were evaluated by a Tukey's test. The following model was used:

$Yijk = \mu + Ti + Aj + eijk$

Where: Y is the j^{th} observation, μ is the overall mean, T is the i^{th} treatment; A is the j^{th} addition and e_{ij} is the error, which is an independent random variable. The interaction between factors was tested, but it resulted not significant for any variable.

For the sensorial data the following model was used:

$Yijkl = \mu + Ai + Pj + Tk + eijkl$

Where: Y is the j^{th} observation, μ is the overall mean, A is the i^{th} addition; P is the j^{th} panelist, T is the k^{th} treatment and e_{ijk} is the error, which is an independent random variable.

Table 1. Physio-chemical parameters and instrumental color in fresh and cooked Cinta Sense burgers as affected by type of omega-3 enrichment

		Fresh burger				Cooked burger					
		C	**M**	**F**	***p (addition)***	**C**	**M**	**F**	***p (addition)***	**SEM**	***p (cooking)***
Water activity		0.95^{b}	0.95^{b}	0.96^{a}	**	0.96^{a}	0.95^{b}	0.96^{a}	**	0.000	n.s.
Moisture (%)		58.44^{abx}	57.33^{bx}	59.51^{ax}	**	56.59^{ay}	54.47^{by}	56.89^{ay}	***	0.605	***
Fat (%)		14.11	13.05	14.01	n.s.	13.65	12.44	13.15	n.s.	2.140	n.s.
Protein (%)		22.09^{a}	20.74^{b}	21.64^{a}	**	22.00^{a}	20.25^{b}	20.24^{b}	**	0.454	n.s.
Cooking loss (%)		-	-	-	-	11.41^{ab}	13.00^{a}	9.70^{b}	**		-
Instrumental color	***L****	58.78^{bx}	60.21^{abx}	61.27^{ax}	**	51.46^{y}	51.12^{y}	52.92^{y}	n.s.	3.906	***
	a*	17.90^{x}	17.27^{x}	17.78^{x}	n.s.	9.66^{y}	10.26^{y}	9.43^{y}	n.s.	0.933	***
	b*	12.08^{y}	12.08^{y}	12.66^{y}	n.s.	16.90^{x}	17.37^{x}	16.92^{x}	n.s.	1.439	***

[a] Means and mean standard errors (SEM) for fresh and cooked burgers with no enrichment (C), enriched with micro-encapsulated fish oil (M) and with bulk fish oil (F).

[b] Different letters (a, b, c) within the same treatment indicate significant differences within addition (control, micro-capsule or fish oil); different letters (x, y) in the same line indicate significant differences between treatments (fresh vs. cooked) within the same addition.

3. RESULTS AND DISCUSSION

Table 1 shows values for physico-chemical and instrumental color parameters in fresh and cooked Cinta Senese burgers.

In fresh meat, significant differences between treatments were found for moisture and protein.

The values for C burger were in agreement with previous studies on Cinta Senese meat (Pugliese *et al.*, 2005). Regarding differences between M and F burgers, they cannot be supported by other scientific evidence, since this trial was the first study on Cinta Senese burger omega-3 enrichment.

Some physico-chemical and color changes were found in fresh burgers, where the F group had the highest L*value, in accordance with Martínez *et al.,* (2009). This can be due to the presence of oil which increased the lightness. As observed for physico-chemical and instrumental color parameters, the type of omega-3 enrichment significantly affected water activity, moisture and protein content. In fresh and cooked burgers, the M samples showed the lowest water activity, moisture and protein values. These effects can be explained by the addition of extra dry matter due to the incorporated microencapsulation material. Thus, in M burgers, 4.7% fish oil microcapsules were added, which contain around 4% moisture, with the rest of the 96% sample being dry matter (Jiménez-Martín *et al*., 2014). Josquin *et al*., (2012) found the same effect, with around 10% lower moisture content in sausages with encapsulated oil than in those with pure fish oil. After cooking, the M burgers showed significantly higher cooking loss than C and F ones; this result could also explain the lowest moisture value found in M burgers after cooking. Heck *et al*., (2017), studied pork burgers added with microencapsulated vegetal oil and observed a higher cooking loss value in control samples compared to modified ones. The type of enrichment did not change the fat content in fresh burgers, as values were similar in the three batches (C, M and F). So, the amount of fish oil added, either microencapsulated or not, was not high enough to cause changes in the total amount of burger fat. In cooked samples, again, M sample showed the lowest moisture, water activity and protein, while they were the most affected by cooking loss. No significant differences were observed due to the type of addition among cooked burgers. The values for moisture, L^* and a^* significantly decreased from fresh to cooked burgers, while b* levels increased. These modifications were expected and in agreement with

previous studies (Baggio *et al.*, 2006), which also reported a significant decrease in the moisture in beef burgers after grilling: while Martínez *et al.,* (2012), observed that moisture, *L** and *a** values were significantly lower in cooked burger patties than in fresh ones. As expected, the lipid content was not modified by the cooking procedure since no fat source was used to cook, as reported also by Baggio *et al.,* (2006) for different grilled meat products.

The results of the physico-chemical analysis with respect to storage and type of addition are shown in Table 2. After chilled storage (T5), moisture and water activity followed the same trend of T0, being higher in M samples than in C and F. As for T0, cooking loss was also found to be higher in M samples with respect to the F ones. Regarding T0, a significant difference was found for L* which was highest in F samples. This is in accordance with results reported by Martínez *et al*., (2012) in hamburgers and by Valencia *et al*., (2008) in fresh pork sausages, where modified samples after a week of chilled storage showed the highest L* scores. After frozen storage none of the above-mentioned parameters were affected by addition, except for a*, which was lower in F samples. Within the same treatment, C and M samples showed higher values for moisture, water activity and a lower cooking loss, compared to T0 burgers. The fatty acid composition of fresh and cooked Cinta Senese burgers from C, M and F batches is displayed in Table 3. The general profile of fatty acids was similar in all samples. MUFAs were the major family of fatty acids, followed by SFAs and with PUFAs being the minor one. Oleic acid (C18:1 n-9) showed the highest percentage, followed, in decreasing order, by palmitic (C16:0), stearic (C18:0), linoleic (C18:2 n-6), palmitoleic (C16:1 n-7) and myristic (C14:0) acids; the rest of fatty acids showed percentages lower than 1%. This is in agreement with other studies on Cinta Senese lipid composition (Pugliese *et al*., 2005). Moreover, no important modifications in the percentages of fatty acids were observed during the cooking procedure. This indicates that the fatty acids in Cinta Senese loin are not very susceptible to change due to the cooking conditions applied in the present study. In different meat products, Baggio *et al*., (2006) found no modification in fatty acid profiles after grilling, whereas Martínez *et al*., (2012), reported significant differences in myristolenic (C14:1), arachidonic (C20:4 n-6) and DHA (C22:6 n-3) acids contained in grilled beef burgers compared to raw samples. Concerning the enrichment-type influence on fatty acid composition, as expected, in C

burgers (fresh and cooked) EPA and DHA were not found; while in M fresh and cooked burgers, significantly higher percentages of EPA and DHA were observed respect to F ones. Since the same omega-3 quantity (1.67 g) has been added in M and F batches, data indicate that maltodextrin and chitosan, which constitute the microcapsule outer layer, provided an effective protection to omega-3 added PUFAs both during manufacturing and cooking.This difference can be explained considering that, in these studies, microcapsules and fish oil were used as a partial replacement of pork back fat; while, in the present work, the fortification was carried out without a previous modification of lipid meat content. Partially according with the studies previously reported, M burgers also showed higher n-3 and lower n-6/n-3 ratio than F and C ones in cooked samples. Indeed, both Keenan *et al*., (2015) and Josquin *et al*., (2012) observed a lower n-6/n-3 ratio in omega-3 enriched samples, but the way fish oil was added (bulk or encapsulated) seemed to make no difference. As occurred with most of the Cinta Senese burger fatty acids, cooking did not significantly influence EPA and DHA percentages. However, in F samples, there was a decreasing tendency of the EPA and DHA percentages from fresh to cooked samples, which was not observed in M burgers. This suggests that microcapsules perform a protective effect on these omega-3 fatty acids during cooking.

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Table 2. Physico-chemical parameters and instrumental color in cooked Cinta Senese burgers as affected by storage (T0 = no storage, T5 = chilled storage, T30 = frozen storage) and type of omega-3 enrichment (C = Control, M = microcapsules and F = fish oil)

		Addition				p (addition)	p (storage)			p (storage * addition)
	Storage	C	M	F	SEM		C	M	F	
	T0	56.59^{y}	54.47^{by}	56.89^{a}	0.469	*	*	*	n.s.	n.s.
Moisture (%)	T5	56.97^{axy}	55.26^{by}	57.24^{a}		*				
	T30	57.87^{x}	57.65^{x}	57.46		n.s.				
	T0	0.96^{ay}	0.96^{by}	0.96^{a}	0.001	**	*	*	n.s.	n.s.
Water activity	T5	0.96^{ax}	0.96^{bxy}	0.96^{a}		**				
	T30	0.96^{xy}	0.96^{x}	0.96		n.s.				
	T0	11.41^{bx}	13.00^{ax}	9.70^{cxy}	0.497	*	n.s.	**	*	*
Cooking loss (%)	T5	10.82^{axy}	9.59abxy	8.51^{by}		**				
	T30	9.57^{y}	10.59^{y}	10.49^{x}		n.s.				
	T0	51.46	51.12	52.92	0.980	n.s.	n.s.	n.s.	n.s.	n.s.
L*	T5	51.30^{b}	52.42^{b}	55.27^{a}		*				
	T30	52.75	52.39	53.12		n.s.				
	T0	9.66	10.26	9.43	0.893	n.s.	n.s.	n.s.	n.s.	n.s.
a*	T5	9.80	8.85	8.53		n.s.				
	T30	9.12^{ab}	11.39^{a}	8.49^{b}		*				
	T0	16.90	17.37	16.92	0.681	n.s.	n.s.	n.s.	n.s.	n.s.
b*	T5	15.51	15.80	15.48		n.s.				
	T30	16.13	17.17	16.24		n.s.				
Fat (%) on a wet basis	T0	13.65	12.44	13.15	0.427	n.s.	n.s.	n.s.	n.s.	n.s.

	T5	14.23	13.46	14.15		n.s.					
	T30	13.56	12.93	13.28		n.s.					
	T0	20.71^{y}	20.25^{y}	20.24^{y}	0.245	n.s.	***	**	***	n.s.	
Protein (%) on a wet basis	T5	20.76^{y}	20.25^{y}	20.73^{y}		n.s.					
	T30	22.60^{x}	22.45^{x}	22.74^{x}		n.s.					

[a] Means and mean standard errors (SEM) for burgers with no enrichment (C), enriched with micro-encapsulated fish oil (M) and with bulk fish oil (F), cooked after different storage conditions (T0=no storage, T5=chilled storage, T30=frozen storage).
[b] Different letters in the same row (a, b, c) indicate significant differences (at least $p < 0.05$) through addition within the same storage conditions.
[c] Different letters in the same column (x, y, z) indicate significant differences (at least $p < 0.01$) regarding storage.

Table 3. Fatty acid composition (expressed as percentage of fatty acid methyl esters) in fresh and cooked Cinta Senese burgers as affected by type of omega-3 enrichment.

	Fresh burger				Cooked burger					
%	**C**	**M**	**F**	***p (addition)***	**C**	**M**	**F**	***p (addition)***	SEM	***p (cooking)***
C12	0.11	0.11	0.11	n.s.	0.12	0.11	0.11	n.s.	0.001	n.s.
C14	1.82	1.96	1.97	n.s.	2.04	1.93	1.84	n.s.	0.059	n.s.
C14:1	0.04	0.04	0.04	n.s.	0.04^{ab}	0.03^{b}	0.05^{a}	n.s.	0.000	n.s.
C15	0.05	0.05	0.06	n.s.	0.05	0.06	0.05	n.s.	0.000	n.s.
C16	27.91	28.48	29.46	n.s.	29.86	28.77	28.56	n.s.	1.679	n.s.
C16:1	3.14	3.15	3.03	n.s.	3.25	3.13	3.08	n.s.	0.057	n.s.
C17	0.21	0.22	0.23	n.s.	0.22^{b}	0.22^{ab}	0.22^{a}	n.s.	0.000	n.s.
C17:1	0.22	0.20	0.20	n.s.	0.21	0.21	0.21	n.s.	0.000	n.s.
C18	10.45	10.96	11.64	n.s.	10.89	11.28	11.31	n.s.	1.258	n.s.
C18:1 n-9	43.55	42.42	41.28	n.s.	41.54	41.84	42.31	n.s.	1.690	n.s.
C18:2 n-6	10.29	10.01	9.72	n.s.	9.75	9.91	9.93	n.s.	0.170	n.s.
C18:3 n-6	0.02	0.02	0.03	n.s.	0.02	0.03	0.03	n.s.	0.000	n.s.
C18:3 n-3	0.56	0.54	0.52	n.s.	0.52^{b}	0.54^{a}	0.54^{ab}	n.s.	0.001	n.s.
C20	0.12	0.13	0.14	n.s.	0.12	0.13	0.13	n.s.	0.001	n.s.
C20:1	0.70	0.73	0.67	n.s.	0.61	0.74	0.71	n.s.	0.007	n.s.
C20:2	0.37	0.36	0.34	n.s.	0.32	0.36	0.37	n.s.	0.001	n.s.
C21	0.07	0.05	0.07	n.s.	0.06	0.07	0.07	n.s.	0.000	n.s.
C20:4 n-6	0.32	0.34	0.31	n.s.	0.33	0.39	0.37	n.s.	0.000	n.s.
C20:3 n-3	0.07	0.07	0.07	n.s.	0.06	0.07	0.07	n.s.	0.000	n.s.

C20:5 n-3	ND[b]	0.07[a]	0.05[a]	***	ND[c]	0.07[a]	0.03[b]	***	0.000	n.s.
C22:6 n-3	ND[b]	0.09[a]	0.06[ab]	**	ND[b]	0.10[a]	0.03[b]	***	0.001	n.s.
SFA	40.73	41.96	43.68	n.s.	43.36	42.59	42.28	n.s.	3.371	n.s.
MUFA	47.64	46.53	45.22	n.s.	45.64	45.94	46.36	n.s.	2.026	n.s.
PUFA	11.63	11.51	11.10	n.s.	11.00	11.47	11.37	n.s.	0.243	n.s.
∑n-6	10.63	10.37	10.06	n.s.	10.10	10.33	10.33	n.s.	0.179	n.s.
∑n-3	0.63[b]	0.77[a]	0.70[ab]	**	0.58[c]	0.78[a]	0.67[b]	***	0.003	n.s.
n-6/n-3	16.89[a]	13.53[b]	14.52[b]	***	17.45[a]	13.29[c]	15.35[b]	***	0.630	n.s.
SFA/PUFA	0.69	0.72	0.78	n.s.	0.77	0.74	0.73	n.s.	0.003	n.s.

[1] Means and mean standard errors (SEM) for fresh and cooked burgers with no enrichment (C), enriched with microencapsulated fish oil (M) and with bulk fish oil (F).
[2] Different letters (a,b,c) within the same treatment indicate significant differences after addition (control, micro-capsule or fish oil); different letters (x,y) in the same line indicate significant differences between treatments (fresh vs. cooked) within the same addition.
[3] ND: not detected.

Table 4 shows the fatty acid profiles of C, M and F burgers with regard to storage. The influence of storage on the FA profile of the burgers was very limited. At T5, α-linolenic acid (C18:3 n3), arachidonic acid and eicosatrienoic acid (C20:3 n-3) were observed to be the highest in the M samples. At T5, M samples preserved the highest percentage of EPA, DHA and omega-3 FA with respect to both C and F burgers. However, in F samples, frozen storage resulted in a better preservation of EPA, DHA and consequently, the omega-3 total content increased if compared to chilled storage. On the contrary, in M burgers, frozen storage determined a loss in DHA and omega-3 with respect to EPA content, if compared to chilled storage. This is probably due, as suggested by Keenan *et al*., (2015), to a number of possible mechanisms occurring during the spray-drying process which, combined with the larger surface of microencapsulated fish oil, in comparison to bulk oil, as well as the longterm storage (30 days), could have promoted the omega-3 degradation. Total PUFA contents were also positively modified by addition at T0 and T5, both in M and F burgers, though, due to the limited amount of fish oil added both as microcapsules and bulk oil, the PUFA improvement consisted only in a 0.4-0.8% increase in M samples and 0.2-0.4% in F samples. Nevertheless, thanks to the fish oil FA profile, rich in omega-3 PUFA, a small amount of it was able to increase the omega-3 content of enriched samples and consequently, to significantly reduce the omega6/omega3 ratio averagely from 17 to 12-13 in M samples and to 14-15 in F samples. These scores are still far from the recommendations of a 4/1 ratio (Wood *et al*., 2008), however, combining the omega-3 enrichment with a partial replacement of fat has already shown promising results in reducing SFA meat contents and lowering omega-6/omega-3 ratios (Josquin *et al*., 2012; Keenan *et al*., 2015).

Table 4. Fatty acid composition (expressed as percentage of fatty acid methyl esters) in cooked Cinta Senese burgers as affected by storage (T0 = no storage, T5 = chilled storage, T30 = frozen storage) and type of omega-3 enrichment (C = Control, M = microcapsules and F = fish oil).

		Addition					p (storage)			p (addition * treatment)
%	**Storage**	**C**	**M**	**F**	***p (addition)***	**SEM**	**C**	**M**	**F**	
C18	T0	10.89	11.28	11.31	n.s.	0.403	n.s.	n.s.	n.s.	n.s.
	T5	11.12	11.68	11.34	n.s.					
	T30	11.11	11.27	10.59	n.s.					
C18:1	T0	41.54	41.84	42.31^{y}	n.s.	0.572	n.s.	n.s.	n.s.	n.s.
	T5	42.13	43.44	42.86	n.s.					
	T30	42.79	42.64	44.02^{x}	n.s.					
C18:2 n-6	T0	9.75	9.91	9.93	n.s.	0.129	n.s.	n.s.	n.s.	n.s.
	T5	9.88	10.23	9.95	n.s.					
	T30	10.03	10.09	10.18	n.s.					
C18:3 n-6	T0	0.02^{b}	0.03^{ab}	0.03^{a}	*	0.002	n.s.	n.s.	n.s.	n.s.
	T5	0.03	0.02	0.03	n.s.					
	T30	0.03	0.03	0.03	n.s.					
C18:3 n-3	T0	0.52	0.54	0.54	n.s.	0.009	n.s.	n.s.	n.s.	n.s.
	T5	0.53^{b}	0.56^{a}	0.53^{b}	*					
	T30	0.54	0.55	0.55	n.s.					
C20:4 n-6	T0	0.33^{b}	0.39^{a}	0.37^{ab}	*	0.017	n.s.	n.s.	n.s.	n.s.
	T5	0.35^{b}	0.42^{a}	0.37^{b}	**					
	T30	0.37	0.38	0.40	n.s.					
C20:3 n-3	T0	0.06^{y}	0.07^{y}	0.07	n.s.	0.005	n.s.	n.s.	*	n.s.
	T5	0.07^{by}	0.08^{ax}	0.07^{ab}	*					

	T30	0.08^{x}	0.07^{y}	0.08	n.s.					
C20:5 n-3	T0	0.00^{y}	0.07^{a}	0.03^{by}	***	0.005	*	n.s.	*	*
	T5	0.01^{cxy}	0.07^{a}	0.04^{bxy}	***					
	T30	0.02^{bx}	0.07^{a}	0.06^{ax}	***					
C22:6 n-3	T0	0.00^{c}	0.10^{ay}	0.03^{by}	***	0.013	n.s.	n.s.	*	*
	T5	0.00^{c}	0.15^{ax}	0.05^{bxy}	*					
	T30	0.00^{b}	0.10^{ay}	0.08^{ax}	***					
SFA	T0	43.36	42.59	42.28^{x}	n.s.	0.747	n.s.	n.s.	n.s.	n.s.
	T5	42.63	40.60	41.69	n.s.					
	T30	41.72	41.67	40.01^{y}	n.s.					
MUFA	T0	45.64	45.94	46.36^{y}	n.s.	0.586	n.s.	n.s.	n.s.	n.s.
	T5	46.17	47.44	46.89^{y}	n.s.					
	T30	46.83	46.69	48.21^{x}	n.s.					
PUFA	T0	10.99^{by}	11.47^{ay}	11.37^{a}	**	0.173	n.s.	n.s.	n.s.	n.s.
	T5	11.20^{by}	11.96^{ax}	11.42^{ab}	*					
	T30	11.45^{x}	11.65^{x}	11.78	n.s.					
N6	T0	10.10	10.33	10.33	n.s.	0.139	n.s.	n.s.	n.s.	n.s.
	T5	10.25^{b}	10.68^{a}	10.35^{ab}	*					
	T30	10.43	10.49	10.61	n.s.					
N3	T0	0.58^{cx}	0.78^{ay}	0.67^{ay}	***	0.024	*	n.s.	*	*
	T5	0.60^{bxy}	0.86^{ax}	0.69^{by}	***					
	T30	0.64^{bx}	0.78^{ay}	0.77^{ax}	***					
N6/N3	T0	17.45^{ax}	13.29^{cy}	15.35^{bx}	***	0.303	*	n.s.	*	*

	T5	17.04^{axy}	12.43^{cy}	14.99^{bxy}	***					
	T30	16.32^{ay}	13.49^{bx}	13.90^{by}	***					
SFA/UFA	T0	0.77	0.74	0.73^{x}	n.s.	0.021	n.s.	n.s.	n.s.	n.s.
	T5	0.74	0.69	0.72^{x}	n.s.					
	T30	0.72	0.72	0.67^{y}	n.s.					

[a]Different letters in the same raw (a, b, c) indicate significant differences ($p < 0.05$) through addition within the same storage conditions. [b]Different letters in the same column (x, y, z) indicate significant differences (at least $p < 0.05$) with storage. [c]The following FAs were detected, but not reported in the table: lauric acid, miristic acid, miristoleic acid, pentadecaenoic acid, palmitic acid, palmitoleic acid, margaric acid, optadecenoic acid, steric acid,arachic acid, gondoic acid and gadoleic acid

Figure 1 shows the lipid oxidation of fresh versus cooked burgers from C, M and F batches. In fresh samples, no differences in oxidation levels were detected. However, the effect of enrichment type strongly impacted cooked samples, with the highest TBAR values for F samples. M burgers, despite the type of addition, showed similar oxidation levels to C. In accordance with our results, Jiménez-Martín *et al.*, (2016a) observed the highest TBAR values in nuggets fortified with bulk fish oil, while microencapsulated and control ones had significantly lower values. These results were also supported by the lower levels of hexanal and nonanal (two additional lipid oxidation markers) in encapsulated-enriched nuggets. Furthermore, Josquin *et al.*, (2012) working with fish

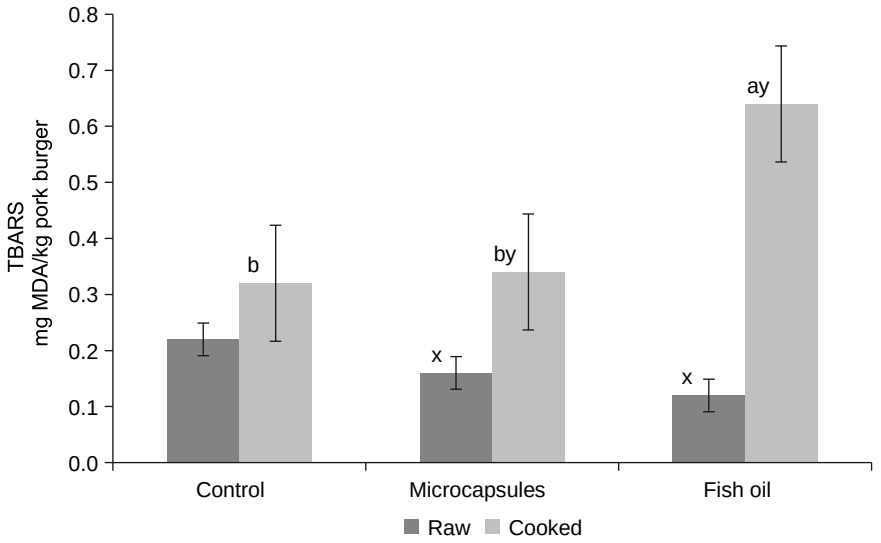


Figure 1. Lipid oxidation in raw and cooked Cinta Senese burgers as affected by the omega-3 enrichment type (control, micro-capsules, fish oil).
[a] Different letters (a,b) within the same treatment (raw or cooked) indicate significant effect ($p < 0.05$) of omega-3 enrichment type; different letters (x,y) within the same addition indicate significant effect ($p < 0.05$) of treatment (raw or cooked).

oil, reported the lowest TBAR content in fermented sausages enriched with encapsulated oil compared to those in the control and bulk oil added ones. The treatment, as expected, significantly increased TBARs from fresh to cooked samples in M and F batches. This is due to the high temperature boosting effect on the oxidation processes taking place during cooking (Valencia *et al.*, 2008; Martínez *et al.*, 2012). Nevertheless, the extent of

lipid oxidation was not the same for all batches. The increase in TBAR values from fresh to cooked samples was more marked in the F batch than in the C and M ones, which, again, indicates that microencapsulated omega-3 fatty acids were protected during cooking.

Figure 2 reports the TBAR scores with respect to addition and storage. M samples were the least oxidized, with values similar to C samples, after both chilled and frozen storage. This is in accordance with the observations made by Valencia *et al*., (2008) on fish oil added burgers and by Jiménez-Martín *et al*., (2016a) on chicken nuggets enriched with microencapsulated and bulk fish oil after frozen storage. However, other authors have obtained contrasting results, showing how, in some cases, the spray-drying technique, mainly due to its elevated temperature, could negatively affect the oxidation of the encapsulated fish oil drops (Pelser *et al*., 2007; Keenan *et al*., 2015). Nevertheless, for both M and F, lipid oxidation scores were far below the perception threshold of 2.00 mg

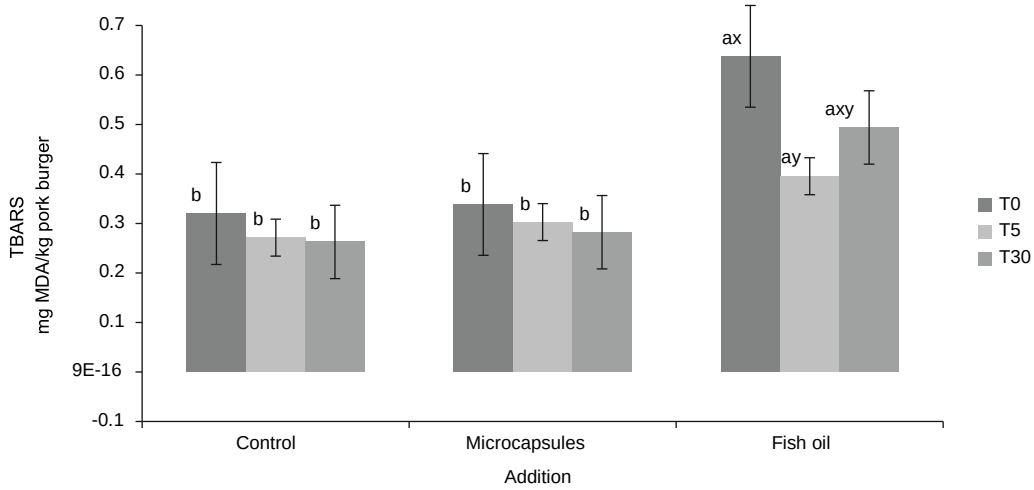


Figure 2. Lipid oxidation at T0, T5 and T30 in Cinta Senese burgers as affected by the type of omega-3 enrichment.

[a]Different letters (a, b, c) within the same storage (T0 , T5 , T30) indicate significant differences (at least $p < 0.05$) among addition types.

[b]Different letters (x, y, z) within the same addition type (Control, Microcapsules, Fish oil) indicate significant differences at least $p < 0.05$) with storage (T0 , T5 , T30).

MDA/Kg of product reported by Greene and Cumuze (1982) as the detectable level perceivable by the majority of meat consumers.

Results from the quantitative-descriptive sensory analysis of cooked burgers are shown in Figure 3. The type of omega-3 enrichment led to significant differences mainly in the following sensory attributes: greasy appearance, odor intensity, cooked meat odor, oiliness, and cooked meat flavor. At T0 and T30, M group showed the lowest scores with respect to C and F burgers. On the contrary, at T5, M burgers showed the best score for those attributes, while F burgers had the lowest. Very few studies on meat products enriched with microencapsulated fish oil are available. Some authors observed no differences in the sensorial characteristics of fermented sausages (Pelser *et al*., 2007) or chicken nuggets (Jiménez-Martín *et al.*, 2016a) enriched with microencapsulated oil, neither for sausages (Josquin *et al.*, 2012) or pork burgers (Martínez *et al*., 2012) added with bulk fish oil.

However, in enriched burgers, Keenan *et al*., (2015) reported the presence of off odors and flavors described as 'fishy' or not 'native' by panelists, who, partially in accordance with our results, gave higher acceptability scores to control samples with respect to added ones, regardless of the type of omega-3 enrichment. In fact, together with the oxidation issue, another pivotal problem linked to the use of fish oil for food enrichment is its peculiar odor and flavor, which, being easily perceivable, can negatively impact the enriched product's sensory characteristics. This problem can be partially avoided since several methods have been developed in order to deodorize the fish oil (Garg *et al*., 2006) so that, in most cases, as well as in this work, enrichment is carried out using deodorized fish oil. Nevertheless, as reported before, this is not always enough to fully protect the enriched product from fishy aroma and taste.

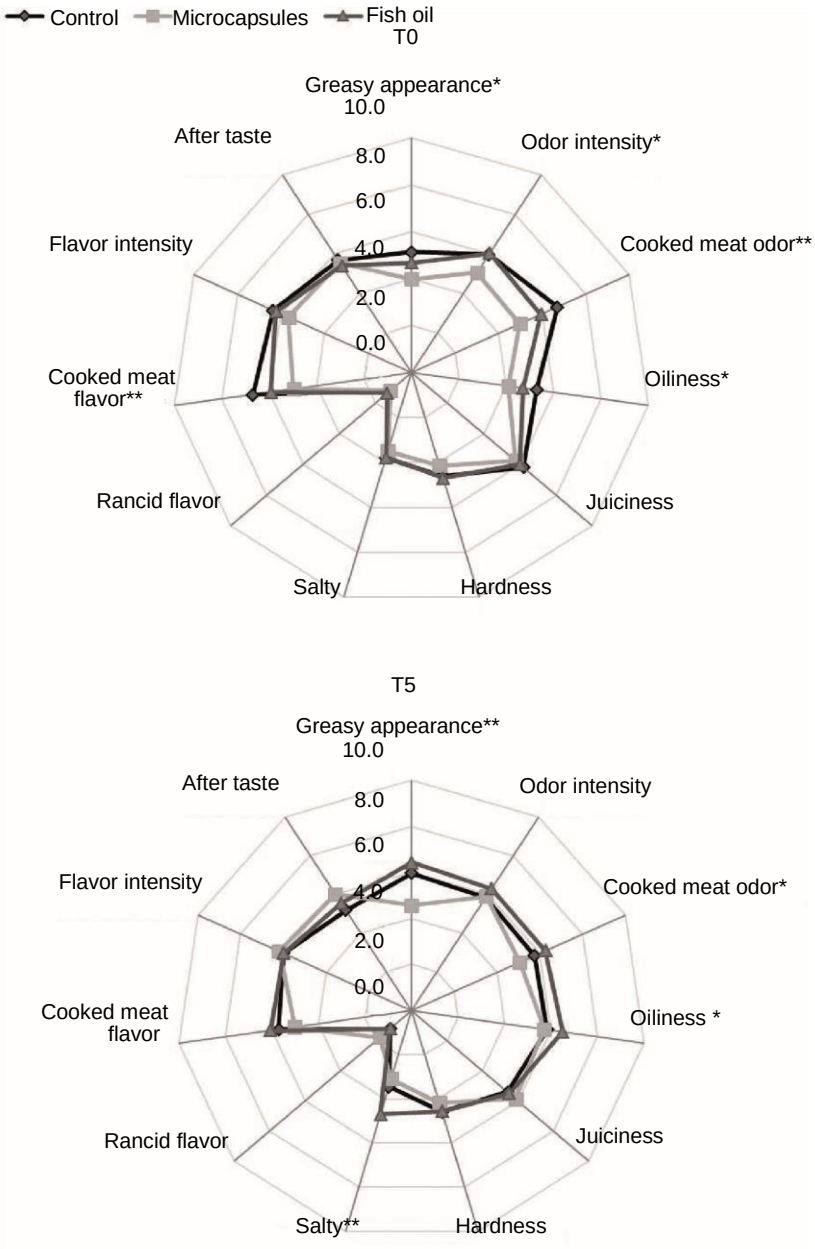
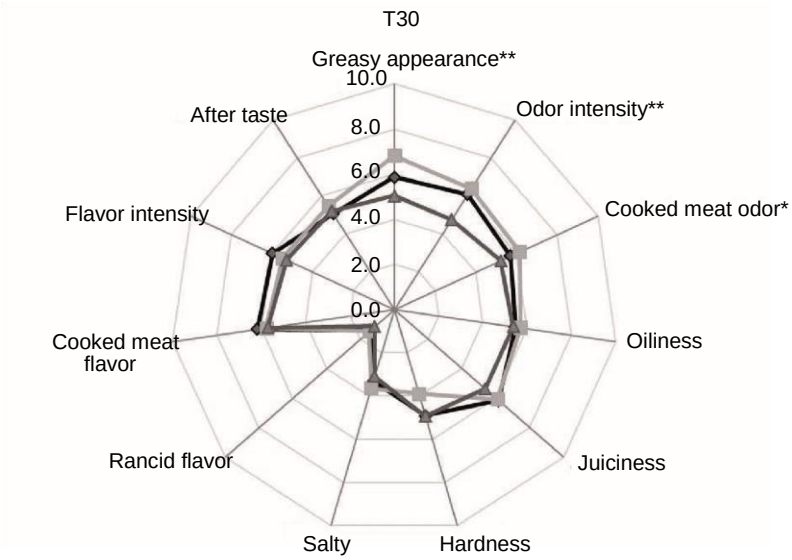


Figure 3. Results of the sensory analysis of Cinta Senese cooked burgers as affected by type of omega-3 enrichment (control ( ), micro-capsules ( ), fish oil ( )) at T0, T5 and T30. The asterisks ((*) $p < 0.05$, (**) $p < 0.01$) indicate significant effects of the omega-3 enrichment type on the burgers' sensory characteristics.

Figure 3. (*Continued*)



4. CONCLUSIONS

Fish oil addition, both with bulk and encapsulated fish oil, has been found to be suitable to fortify Cinta Senese burgers with EPA+DHA. M fortification did not affect burger instrumental color, total lipid content or fatty acid profile, except for EPA and DHA. Indeed, their content was found to be greater in M burgers after cooking and after storage with respect to F ones. This is in accordance with lipid oxidation scores, which were comparable for C and M, while F samples were always the most oxidized. Finally, sensorial analyses results indicate that chilled storage is more suitable for products added with microcapsules, whereas, for bulk fish oil enriched burgers, frozen storage is more appropriate. To sum up, multi-layer microcapsules produced by spray-drying were observed to be effective in enriching poorly provided foods with omega-3 PUFAs. In addition, producing omega-3 enriched burgers from Cinta Senese pigs, might improve the profitability of this local and high-quality pig production system.

ACKNOWLEDGEMENTS

This project received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 634476 (Project acronym: TREASURE).

The content of this paper reflects only the author‘s view and the European Union Agency is not responsible for any use that may be made of the information it contains.

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Chapter 3

Effect of natural antioxidants from grape seed and chestnut in combination with hydroxytyrosol, as sodium nitrite substitutes in Cinta Senese dry-fermented sausages

Published on Meat Science as:

Aquilani, C., *et al.* (2018) 'Effect of natural antioxidants from grape seed and chestnut in combination with hydroxytyrosol, as sodium nitrite substitutes in Cinta Senese dry-fermented sausages', *Meat Science*, 145, pp. 389–398. doi: 10.1016/j.meatsci.2018.07.019.

Authors: **Aquilani C.**, Sirtori F., Flores M., Bozzi R., Lebret B., Pugliese C.

Personal contribution: Physical, chemical and sensorial analysis, collaboration in experimental design and statistical elaboration, writing and editing of the paper as corresponding author.

Meat Science 145 (2018) 389–398

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Effect of natural antioxidants from grape seed and chestnut in combination with hydroxytyrosol, as sodium nitrite substitutes in Cinta Senese dry-fermented sausages

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ARTICLE INFO

Keywords:
Volatile compounds
Meat quality
GC-olfactometry
Local pig breed
Lipid oxidation
Pork products

ABSTRACT

Dry-fermented pork sausages, from Cinta Senese local breed, were manufactured replacing sodium nitrite (NIT) with two mixtures of natural antioxidants consisting of: i) grape seed extract and olive pomace hydroxytyrosol (GSE); ii) chestnut extract and olive pomace hydroxytyrosol (CHE). The effects on physical-chemical, aromatic and sensory traits, as well as the microbiological safety, were tested. Nitrite replacement lowered the pH in GSE and CHE samples and resulted in several differences in physical traits between CHE and NIT samples. *Listeria monocytogenes, Salmonella* and *Clostridium botulinum* were not found in any samples. GSE and CHE mixtures showed a slightly lower antioxidant activity. Volatile profile showed a similar aromatic profile among the three treatments with differences mainly to abundance of the single compounds, indicating that replacement of nitrite by natural antioxidants did not affect the overall aroma profile, as outlined by olfactometry results. In addition, the replacement did not affect the overall acceptability, except for color-related traits, underscored in GSE and CHE products.

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https://doi.org/10.1016/j.meatsci.2018.07.019
Received 26 April 2018; Received in revised form 13 July 2018; Accepted 13 July 2018
Available online 17 July 2018

1. Introduction

Dry cured meat products are typical of the Mediterranean area and they represent a high-value production in European countries, considering that curing process allows extension of meat shelf-life (Marco, Navarro, & Flores, 2006) and leads to typical pork products with specific eating quality and regional identity (Pugliese & Sirtori, 2012). In Southern Europe, salami and dry-fermented sausages, are generally characterized by slowly air-drying and mold-ripening (Flores, 1997). This curing process leads to peculiar characteristics and flavors that are widely appreciated by consumers; but is also related to longer curing times that may cause higher lipid oxidation levels. Moreover, natural fermentation, avoiding the addition of lactic acid-producing starter cultures, is more susceptible to the growth of harmful bacteria, such as Listeria monocytogenes or Clostridium botulinum (Lücke, 2000). Thus, to avoid a severe deterioration of nutritive and organoleptic attributes, as well as to ensure food safety, several synthetic food preservatives are commonly included. Among them, the most used are nitrites and nitrates (Hammes, 2012). Nitrite positively affects color, inhibits the growth of pathogenic bacteria, contributes to the development of typical cured meat flavor and delays oxidative rancidity (Marco et al., 2006). Despite their effectiveness as curing agents, the nitrite/nitrate intake represents a risk to human health, i.e. the formation of carcinogenic nitrosamines is one of the most current concerns (De Mey, De Maere, Paelinck, & Fraeye, 2017). Several studies have focused on nitrate/nitrite reduction or substitution (Özvural & Vural, 2014; Pateiro, Bermúdez, Lorenzo, & Franco, 2015; Purriños, García Fontán, Carballo, & Lorenzo, 2013), but the main issue remains finding an alternative able to address the multiple activities they perform. Up until now, most of the alternatives proposed are plant extracts, largely obtained from agricultural by-products. These compounds are very rich in polyphenols, flavonoids and terpenoids and are able to perform a double antioxidant-antimicrobial functions (Falowo, Fayemi, & Muchenje, 2014; Hygreeva, Pandey, & Radhakrishna, 2014; Shah, Bosco, & Mir, 2014). These compounds might also constitute a great opportunity to exploit agricultural by-products, which otherwise would be wasted. The aim of this study was to assess the feasibility of producing dry-fermented sausages by replacing sodium nitrite with natural antioxidants while trying to maintain quality traits. Grape seed extract, chestnut extract and hydroxytyrosol (extracted from defatted olive pomace), were chosen due to their

great availability as by-products of important Tuscan agricultural products. Moreover, among the investigated plant extracts, they have shown an interesting potential both for antioxidant activity and microbial inhibition. This innovation also aimed to valorize Cinta Senese, a local pig breed strongly linked to the Tuscan region.

Table 1. Phenolic profile of olive pomace and defatted grape seed and chestnut extracts.

Olive pomace (hydroxytyrosol)		**Grape seed**		**Chestnut**	
(g/L)		**(mg/g)**		**(mg/g)**	
Hydroxytyrosol	11.65	Gallic acid	0.01	Vescalin	9.34
Tyrosol & hydroxytyrosol derived compounds	15.13	Catechin B3 (dimers)	2.22	Castalin	8.99
Verbascosid	5.84	Catechin	11.07	Pedunculagin I	3.88
		Catechin (trimers)	3.21	Monogalloil glucose I	3.58
		Catechin B6 (dimers)	2.61	Gallic acid	18.50
		Catechin B2 (dimers)	5.37	Monogalloil glucose II	2.73
		Epicatechin	13.62	Roburin D	10.51
		Catechina trimer	3.71	Vescalagin	32.15
		Epicatechin gallate (PM 730)	6.65	C-glucoside tergallic dehydrate	2.73
		Epicatechin gallate (PM 442)	6.10	Castalagin	31.03
		Oligomers (tetramers)	54.88	Digalloil glucose I	10.03
		Epicatechin gallate (PM 882)	180.65	Digalloil glucose II	2.09
		Epicatechin gallate oligomers (trimers)	382.97	Hydrolyzable tannin m/z 1085	8.05
		Epicatechin gallate oligomers (trimers)	149.66	Trigalloil glucose I	4.61
				Trigalloil glucose II	6.74
				Tetragalloil glucose	2.05
				Ellagic acid	4.08

2. Materials and methods

2.1. Antioxidant mixtures

The natural antioxidants employed in the present studies were provided by Phytolab (Sesto Fiorentino, Florence, Italy). They consisted of grape seed and chestnut extracts, tocopherol and hydroxytyrosol (extracted by defatted olive pomace). The manufacturer provided the phenolic profile (Table 1), total phenolic content and antiradical scavenging activity (EC_{50}) (Table 2) of each extract. The grape seed and chestnut extracts were combined with the same amount of hydroxytyrosol and tocopherol to form two different mixtures; grape seed (GSE) and chestnut (CHE) mixtures.

2.2. Sausages manufacturing

In an industrial plant (Azienda Agricola Savigni, Pistoia, Italy), 24 kg of pork lean and 6 kg of subcutaneous backfat from Cinta Senese pig breed were minced and equally divided in three batches. Salt (23 g/ kg), sucrose (35 g/kg) and black pepper (0.2 g/kg) were added to each batch following the recipe traditionally used by the manufacturer. Thirty ppm of sodium nitrite (E250) were added to the first batch to constitute the control (NIT). In second batch, 10 g/kg of GSE mixture were used to replace sodium nitrite, while 10 g/kg of CHE were added to the third batch. Sausages were weighed, dried at 28 °C and RH 85% for 4 days and then ripened 21 days (T 13 °C, RH 70%).

Table 2. Total phenolic content and radical scavenging activity of natural antioxidant constituting the mixtures.

	Total phenolic content	**Antiradical scavenging activity (EC_{50})**
Grape seed extract	822.709 (mg/g)	0.147
Chestnut extract	161.091 (mg/g)	0.085
Olive pomace (hydroxytyrosol)	32.62 (g/l)	0.196
α-tocopherol	–	0.184

Once ripened, six samples of each batch were collected, pH, color, and processing loss were immediately measured. Samples were vacuum packed and stored at −80 °C for physical, chemical and aromatic analysis. Another 3 samples of each batch were stored at 4 °C to be employed for sensory analysis the following day. This design was replicated to have two totally independent batches for each treatment.

2.3. Physical, chemical and microbiological parameters

At the end of ripening, physical parameters were assessed on 12 samples of each batch (6 for each replication). Sausage pH was measured at room temperature (20 °C) using a pH meter Crison GLP21 (Barcelona, Spain), the instrument was introduced in a sausage portion. Color (L*, a* and b*) was determined by a Minolta Chromameter CR200 (Tokyo, Japan) immediately after slicing. a_w was measured following the method ISO 21807:2004. Two 10 mm-thick and 10 mm-width slices of each sample, were cut and immediately analyzed at room temperature (22 °C), using a Zwick Roell Z2.5 apparatus (Ulm, Germany) with a loading cell of 1 kN at the crosshead speed of 1 mm/s. Texture profile analysis (TPA) was performed assessing the following parameters: hardness, cohesiveness, gumminess, springiness and chewiness. Moisture was determined by lyophilizing to constant weight 40 g of sample, according to AOAC methods (1990). Weight loss was measured as the difference between weight at time zero and end of ripening (after 24 days). Total protein, fat and ash contents were determined following AOAC (1990) methods. Lipid oxidation was determined according Vyncke (1970), using a PerkinElmer Lambda EZ150 spectrophotometer (Waltham, MA, USA). Results were expressed as mg of malondialdehyde (MDA)/kg of samples. Fatty acids were determined using a Varian GC-430 apparatus equipped with a flame ionization detector (FID) (Palo Alto, CA, USA) as reported by Sirtori et al. (2015). The individual methyl esters were identified by their retention time using an analytical standard (F.A.M.E. Mix, C8-C22 Supelco 18,9201AMP). Response factors based on the internal standard (C19:0) were used for quantification and results were expressed as mg/100 g of sample. The fatty acid content was reported as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Microbiological analyses were carried out in an external accredited laboratory to determine the products' safety. The following bacteria were investigated: Escherichia coli (ISO 16649–2:2001), Listeria monocytogenes (UNI EN ISO 11290–1:2005), coagulase positive Staphylococcus spp. (UNI EN ISO 6888–1:2004), Clostridium botulinum (ISO 15213:2003) and Salmonella spp. (UNI EN ISO 6579:2008).

2.4. Volatile compounds analysis

2.4.1. Gas chromatography-mass spectrometry analysis (GC–MS)

Solid-phase microextraction (SPME) and GC–MS analysis were performed following the method described by Corral, Salvador, and Flores (2013) using a 85 μm Carboxen/Polydimethylsiloxane (CAR/ PDMS) fiber (Supelco, Bellefonte, PA) installed in a Gerstel MPS2 multipurpose sampler (Gerstel, Germany) and an Agilent HP 7890 series II GC with an HP 5975C mass selective detector (Hewlett-Packard Palo Alto, CA, USA). The volatile compounds (VOCs) detected were identified by comparison with mass-spectra from the library database (Nist'05), linear retention index (van Den Dool & Dec. Kratz, 1963) and by comparison with authentic standards. The quantification of volatile compounds was done in SCAN mode using either total or extracted ion chromatogram (TIC or EIC) on an arbitrary scale.

2.4.2. Gas chromatrography-olfactometry analysis (GC-O)

A gas chromatograph (Agilent 6890, USA) equipped with an FID detector and sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) was used to analyze aroma compounds extracted by SPME as described by Corral et al. (2013). The detection frequency (DF) method was used to estimate the aromatic impact of each volatile and each assessment was carried out according to Olivares, Navarro, and Flores (2011). Four trained panelists evaluated the odors from the GC-effluent. Each assessor evaluated 3 sausages for a total of 12 assessments, the final DF was obtained by summing the 12 sniffings. The detection of an odor by less than three assessors was considered noise. Compounds were identified by comparison with mass spectra, with linear retention indices of authentic standards injected in the GC–MS and GC-O, and by coincidence of the assessors' descriptors with those reported by Burdock (2010).

2.5. Sensory analysis

Sensory analysis was carried out in an equipped laboratory by 8 trained panelists using a quantitative-descriptive analysis method. Fourteen attributes (grease appearance, abnormal colors, firmness, color uniformity, redness, cured meat flavor, off odor, salty, rancid, off flavor, hardiness, juiciness, aftertaste, general acceptability) were evaluated, each attribute was scored in a 10 cm non-structured line (Pugliese et al., 2010). Select subjects underwent an introductory session, where the testing procedures and the chosen sensory traits were discussed using two types of comparable commercial products. During three sessions, panelists evaluated a total of 9 sausages (3 samples × 3 treatments)

identified by an alphanumerical code. The sausages were divided in 0.5 cm-thick × 2 cm-diameter slices and two slices of each samples were randomly served to judges at room temperature (20 °C). Panelists were invited to eat a cracker and drink a glass of water between samples.

2.6. Statistical analysis

Data were analyzed by SAS software. Two-way ANOVAs were performed on physical and chemical data according to the following model:

$Yijk = \mu + Ti + Bj + \varepsilon ijk.$

Where μ is the mean, T is the i^{th} treatment, B is the j^{th} batch and ε is the error. For sensory data, effect of panelist was included in the previous model. The interaction between Treatment and Batch factors was tested but being not significant, it was not included in the model.

Volatile compounds data were also analyzed by a multivariate approach to determine the presence of characteristic compounds able to be allocated to samples among different treatments. A stepwise discriminant analysis (SDA) was first used to reduce the space-variables, selecting the subset of variables that better discriminated groups. Canonical discriminant analysis (CDA) were performed using SDA selected variables, resulting in 2 new variables, called canonical functions (CAN1, CAN2). They consisted of a series of canonical coefficients (CC) that indicate the partial contribution of each variable in composing the CANs. The greater the CC, the more the variable contributes to CAN composition.

3. Results and discussion

3.1. Physical, chemical and microbiological parameters

The major foodborne pathogens (Escherichia coli, Listeria monocytogenes, Staphilococcus spp., Clostridium spp. and Salmonella spp.) were absent or below the limit required (Reg CE/2073/05) in all samples (Table 3). Several studies on plant phenolic suggest these components have antimicrobial activity (Fasolato et al., 2016; Kao et al., 2010; Mujić et al., 2014). Further studies are, however, required to determine the effectiveness of the studied antioxidants against the development of the main foodborne pathogens. The a_w values (Table 4) recorded for GSE and CHE, being below 0.89, contributed to control pathogenic organisms' development (Toldrá & Flores,

2014). Moisture, fat, protein and ash contents and weight loss were not affected by treatment and they were in line with values reported for dry-cured sausages (Olivares, Navarro, & Flores, 2015; Ribas-Agustí et al., 2014; Škrlep, Čandek-Potokar, Tomažin, Batorek Lukač, & Flores, 2017), except for weight loss, which was slightly greater in the present study; likely the smaller diameter of samples could have enhanced the water loss during ripening. The pH observed was in the range reported for natural fermented meat products at a similar curing time (Özvural & Vural, 2014; Škrlep et al., 2017); indeed, they were characterized by a higher pH compared to commercial products (Hospital et al., 2015; Montanari et al., 2018).

Table 3. Microbiological safety parameters on Cinta Sense dry-fermented sausages manufactured with natural antioxidant (GSE = grape seed extract; CHE = chestnut extract) as replacement of sodium nitrite (NIT).

	GSE	**CHE**	**NIT**
Escherichia coli	< 10	< 10	< 10
Listeria monocytogenes	–	–	–
Coagulase positive Staphilococcus spp.	< 10	< 10	< 10
Sulfite-reducing bacteria (Clostridium botulinum)	< 10	$1.2\ 10^2$	$0.7\ 10^2$
Salmonella spp.	–	–	–

Results are expressed as ufc/g; the symbol "–" indicates that the organism was not present.

Moreover, significant differences among treatments were found for pH, with lower values observed for GSE and CHE samples, suggesting that Lactobacillus (LAB) growth, that takes place during the first fermentation phase, could be slightly promoted in these products. Indeed, low or nitrites-free sausages showed an increased presence of LAB (Hospital et al., 2015). Growth of LAB was not, however, assessed in the present experiment, and further studies will be required to assess effects of GSE and CHE. Concerning color attributes, L* was not affected by treatment, a* showed significant greater values in GSE and NIT samples than in CHE ones, while b* was significant higher in NIT compared to the modified products. A change in a* was expected considering the role nitrites play in nitrosomyoglobin formation, the characteristic red curing pigment (Hammes, 2012). Since neither chemical composition nor oxidation resulted in significant differences among groups, a different pathway for red color formation in GSE samples should be considered. A stable red color compound called Zn-protoporphyrin, derived from the substitution of heme iron with zinc, has been

observed in Parma ham, an Italian nitrite/nitrate-free ham (Wakamatsu, Nishimura, & Hattori, 2004a).

Table 4. Physical and chemical parameters on Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

	GSE	CHE	NIT	SEM[a]	P[b]
pH 0 days	6.13	6.40	6.35	0.05	n.s.
pH 24 days	6.02b	6.04b	6.10a	0.04	**
a_w	0.89	0.88	0.90	0.01	n.s.
L*	43.21	41.67	42.37	0.67	n.s.
a*	17.22a	15.92b	18.06a	0.41	**
b*	5.31b	4.87b	6.48a	0.21	**
Weight loss (%)	40.23	43.30	42.92	1.35	n.s.
Moisture (%)	30.43	29.97	30.94	0.66	n.s.
Fat (g/100 g dm)	41.82	41.01	41.32	0.33	n.s.
Protein (g/100 g dm)	48.88	49.74	49.14	0.31	n.s.
Ash (g/100 g dm)	8.46	8.69	8.80	0.12	n.s.
TBARs (mg MDA/kg)	1.05	0.98	0.93	0.05	n.s.
SFA (mg/100 g)	8.05	7.57	7.71	0.23	n.s.
MUFA (mg/100 g)	9.72	9.17	9.10	0.25	n.s.
PUFA (mg/100 g)	3.32	3.38	3.47	0.10	n.s.

Values are reported as means of the two replications within the same treatment, where GSE is grape seed extract added group, CHE the chestnut extract added group and NIT the control group added with sodium nitrite.
a Standard error.
b P value of natural antioxidant effect □□p < 0.01, □p < 0.05, different letters in the same row indicate significant differences at p < 0.05.

Up to now, mechanisms leading to its formation are not well-known (Hammes, 2012), but the absence of nitrites, low levels of oxygen, meat endogenous enzymes as well as microorganism, are all factors that may contribute to its formation (Wakamatsu et al., 2004b). Apart from the absence of nitrites, some compounds contained in grape seed extract may have promoted the Zn-protoporphyrin formation for the GSE group, while no formation occurred in the CHE samples. These results are partially in agreement with those reported by Lorenzo, González-Rodríguez, Sánchez, Amado, and Franco (2013), althought their study was conducted on chorizo where pigmentation due to added paprika may have interfered.

According to TPA results (Table 5), cohesiveness, springiness and chewiness were affected by nitrite replacement, being highest in CHE samples, lowest in NIT, whereas GSE samples were similar to both.

Table 5. Texture traits of Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

	GSE	**CHE**	**NIT**	**SEM[a]**	**Pb**
Hardness (N)	104.93	102.82	102.68	6.38	n.s.
Cohesiveness	0.38ab	0.42a	0.35b	0.01	**
Gumminess	39.57	42.93	35.99	2.11	n.s.
Springiness	3.04ab	3.26a	2.70b	0.13	*
Chewiness (N)	120.67ab	139.55a	96.79b	8.36	**

Values are reported as means of the two replications within the same treatment, where GSE is grape seed extract added group, CHE the chestnut extract added group and NIT the control group added with sodium nitrite.
[a] Standard error.
b P value of natural antioxidant effect ** $p < 0.01$, * $p < 0.05$, different letters in the same row indicate significant differences at $p < 0.05$.

Since no differences in moisture and weight loss were found among the treatments, the results obtained were attributable basically to the differences in pH, which, declining, causes the aggregation of myofibrillar proteins and leads to gel formation (Lücke, 2000). The higher pH of NIT samples likely inhibited this phenomenon thus reducing the sausage cohesiveness and chewiness. The results reported are partially in agreement with Lorenzo et al., (2013), who also noticed the highest chewiness values for chorizo with added chestnut extract and ripened for 19 days, compared to the same product manufactured with GSE or synthetic antioxidant (BHT in this case).

The groups did not differ in SFA, MUFA and PUFA contents. Their relative amounts reflect those of fresh pork composition, slightly richer in MUFA than SFA, with PUFA being approximately a third of either SFA or MUFA categories (Škrlep et al., 2017). PUFA can also be considered an indicator of meat oxidative status, due to their double bonds being preferred substrates for oxidative reactions (Pateiro et al., 2015). Results suggest that the natural antioxidants employed were as effective as nitrites in control lipid oxidation during manufacturing and ripening. This is supported by TBARs results, showing no significant differences among treatments, however, further studies will be required to evaluate the antioxidant activity during the shelf-life. Nitrites exert their

antioxidant activity in cured meat by forming the myoglobin-stable compounds and making the iron inaccessible for oxidation (Riazi, Zeynali, Hoseini, Behmadi, & Savadkoohi, 2018); phenolic compounds instead, follow different pathways, acting as hydrogen donors. The phenolic hydroxyl groups intercept the free radicals to form stable end-products, interrupting and avoiding further lipid oxidation, especially of unsaturated FAs (Jayaprakasha, Selvi, & Sakariah, 2003). To the best of our knowledge, few data are available about dry-fermented pork sausages with added natural extracts, but the great variability of these traditional products makes comparisons difficult. The efficacy of hydroxytyrosol in preventing lipid oxidation was reported by Cofrades et al. (2011) for n-3 enriched frankfurters, while Lorenzo et al., (2013) observed comparable TBARS values in Spanish chorizo with added BHT, grape seed extract or chestnut extract.

3.2. Volatile profile and olfactometry

Ninety-one VOCs were identified by HS-SPME-GS-MS (Table 6). The most abundant groups originated from spices (51–61%) and carbohydrate fermentation (30–39%), followed by amino acid degradation (6–7%), while VOCs derived from lipid β-oxidation and lipid oxidation processes represented 1% of total extracted areas.

Among the 14 VOCs related to lipid auto-oxidation, 5 showed significant differences with NIT resulting in the lowest, while GSE and CHE products showed intermediate or higher abundances. These VOCs originate by autocatalytic fat oxidation and involves mostly unsaturated fatty acids. Among the identified VOCs, hexanal is of key importance to better outline the products' oxidation status. The correlation between this compound and lipid oxidation is well-known and its low perception threshold makes hexanal an important contributor to overall aroma (Marco et al., 2006). The higher hexanal content in GSE and CHE samples than NIT is consistent with TBARs results, suggesting greater PUFA oxidation, even if the extent was limited and did not affect the parameters previously examined. The total lipid auto-oxidation values confirmed these differences, with NIT being the lowest, GSE the highest and CHE similar to both, likely related to a higher EC50 for GSE. Hence, even though the phenolic extracts used contributed to maintain lipid oxidation below the perception threshold of rancid flavor, they appeared less effective than nitrites in controlling lipid oxidation. Partially in agreement with this, Purriños et al. (2013) reported grape seed was a less effective

antioxidant in chorizo, but on the contrary, chestnut extract was found to have higher antioxidant activity than BHT.

Table 6. Volatile compounds in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	LRI[a]	RI[b]	GSE	CHE	NIT	SEM[c]	P[d]
Lipid auto-oxidation							
Pentane	500	a	0.59	0.60	0.56	0.19	
Propanal	524	a	0.19	0.13	0.13	0.06	
Isopropanol (45)	539	–	1.20	0.79	1.13	0.40	
Hexane	600	a	0.76	0.74	0.56	0.22	
1-Propanol	613	a	4.90	4.90	3.72	2.37	
Octane	800	a	4.20	3.498	3.90	0.98	
Propanoic acid (74)	814	a	0.14a	0.05b	0.02b	0.05	**
1-Pentanol	827	a	0.34ab	0.43a	0.22b	0.16	*
Hexanal	841	a	2.62a	2.27a	1.36b	0.27	**
1-Hexanol	924	a	0.79	0.75	0.88	0.16	
Decane	1000	a	1.10a	1.26a	0.48b	0.18	**
Dodecane	1200	a	0.96	1.14	0.92	0.37	
Tridecane	1300	a	0.36	0.44	0.34	0.10	
Tetradecane	1400	a	0.18b	0.87a	0.22b	0.16	**
Total			19.79a	16.19ab	14.57b	0.96	**
Spices							
α-Thujene	934	b	24.40	26.07	27.29	6.22	
α-Pinene	940	a	17.84	19.00	17.33	3.68	
Sabinene+β-pinene	986	a	133.28	149.70	145.97	19.83	
β-Myrcene	1003	b	37.19b	53.47a	39.01b	9.25	**
α-Phellandrene (93)	1019	a	8.71b	12.39a	9.41b	2.41	**
3-Carene	1023	a	73.20	86.24	77.49	15.77	
α -Terpinene	1035	a	3.66b	4.54a	3.28b	0.78	*
Unknown (57)	1042	b	0.17	0.16	0.14	0.11	
Limonene	1046	a	307.31b	401.19a	289.74b	60.75	**
β-Phellandrene (93)	1051	b	13.98b	19.28a	14.42b	3.74	*
p-Cymene (119)	1052	b	8.49	9.078	8.98	2.73	
β-Ocimene	1067	b	1.18b	1.87a	1.15b	0.44	**
4-Carene	1073	b	0.21	0.27	0.22	0.12	
Unkwon	1075	b	3.21b	4.28a	3.16b	0.93	*
Styrene	1091	a	2.20	2.43	2.34	0.31	

Table 6. Volatile compounds in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	**LRI[a]**	**RI[b]**	**GSE**	**CHE**	**NIT**	**SEM[c]**	**P[d]**
Terpene	1099	b	0.95	1.46	1.35	0.31	
Terpinolene	1101	b	4.64b	6.48a	4.46b	1.20	**
Unknown (93)	1120	b	0.51	0.55	0.56	0.11	
Linalool (93)	1150	a	0.28b	0.33a	0.32ab	0.04	*
β-Terpinene/γ-Terpinene	1158	b	1.4	1.37	1.43	0.24	
4-Terpineol	1231	a	2.14	2.31	2.12	0.29	
Estragole	1249	a	1.28	1.33	1.20	0.21	
α -terpineol	1256	a	0.82	0.91	0.88	0.13	
δ-Elemene	1342	b	3.58a	4.19a	2.74b	0.66	**
α-Cubebene	1348	b	0.85a	0.92a	0.68b	0.12	**
Cyclosativene (161)	1402	b	0.02b	0.04a	0.02b	0.00	*
Copaene (161)	1406	b	1.50a	1.42a	1.16b	0.28	*
β-Cubabene (161)	1421	b	0.20a	0.19a	0.16b	0.04	*
β-Elemene (93)	1423	b	0.11b	0.13a	0.09b	0.04	**
α -Bergamotene	1433	b	0.79a	0.91a	0.69c	0.13	**
trans-α-Bergamotene	1450	b	0.67	0.84	0.76	0.18	
β-Caryophyllene	1455	b	107.97a	113.01a	91.99b	13.04	**
α -Caryophyllene	1486	b	4.87a	4.99a	4.19b	0.68	*
Isocaryophillene	1498	b	0.65	0.84	0.57	0.26	
Isolongifolene	1510	b	10.64b	11.05b	25.91a	10.05	**
Valencene	1513	b	0.00b	5.31a	0.00b	1.78	**
γ-Cadinene	1518	b	1.72b	1.89b	2.92a	0.56	**
δ-Cadinene	1529	b	0.96b	1.04b	1.29a	0.20	**
Total			783.55b	984.05a	785.83b	134.67	**
Bacterial metabolism							
Lipid β-oxidation							
2-Pentanone	734	a	5.64	5.96	6.92	1.79	
3-Octanone	1032	a	2.23	2.51	2.63	0.65	
Total			7.87b	8.47ab	9.55a	0.63	*
Esterase activity							
3-Methyl-1-butanol acetate	907	a	0.59	0.49	0.51	0.12	
Carbohydrate fermentation							

Table 6. Volatile compounds in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	**LRI[a]**	**RI[b]**	**GSE**	**CHE**	**NIT**	**SEM[c]**	**P[d]**
Acetaldehyde	462	a	1.26a	0.86b	0.89b	0.25	**
Ethyl alcohol	507	a	6.79a	5.65ab	3.68b	2.23	*
Acetone	529	a	72.92	76.47	72.72	20.58	
2,3-Butanedione	627	a	9.76a	7.47b	7.83b	2.01	*
2-Butanone	632	a	241.96	287.47	332.22	91.70	
2-Butanol	644	a	19.99	20.52	29.19	11.02	
Acetic acid (60)	720	a	11.99	12.19	7.08	5.34	
1-Butanol (56)	727	a	0.28	0.18	0.71	0.67	
2-Pentanol	756	a	0.49	0.43	0.38	0.15	
3-Hydroxy-2-butanone	782	a	124.14a	89.35b	136.91a	31.84	**
2,3-Butanediol (45)	884	a	6.20a	1.50b	3.76b	2.92	**
2,3-Butanediol (45)	892	a	4.17	2.30	2.36	1.98	
Butanoic acid (60)	894	a	0.52a	0.49a	0.18b	0.254808	*
Total			499.97b	489.85b	597.61a	23.69	**
Amino acid degradation							
2-Methylpropanal	595	a	0.54b	0.66a	0.39b	0.17	**
3-Methylbutanal	691	a	11.34	12.43	15.34	4.53	
2-Methylbutanal	701	a	6.18	7.53	8.85	2.88	
Toluene	789	a	51.99	56.94	59.18	6.88	
3-Methylbutanol	795	a	7.39b	8.46b	15.13a	5.92	*
2-Methylbutanol	797	a	1.27	1.44	1.82	5.92	
Pyrrole	845	a	0.31	0.30	0.25	0.05	
2-Methylpropanoic acid	868	a	2.01	1.70	2.70	1.06	
Ethylbenzene (91)	884	a	0.09b	0.18a	0.09b	0.05	**
3-Methylbutanoic acid (60)	942	a	2.52	2.25	2.27	1.05	
2,5-Dimethylpirazine (108)	943	a	0.34	0.34	0.36	0.09	
2-Methylbutanoic acid	948	a	2.32	2.73	2.98	1.25	
2-Acetyl-1-pyrroline	961	a	0.72b	0.91b	1.37a	0.37	**
Methional	986	a	0.16	0.18	0.15	0.02	

Table 6. Volatile compounds in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	**LRI[a]**	**RI[b]**	**GSE**	**CHE**	**NIT**	**SEM[c]**	**P[d]**
Benzaldehyde (106)	1020	a	1.14	1.40	1.27	0.33	
Benzeneacetaldehyde	1110	a	6.61a	4.14b	2.72b	3.17	*
Tetramethylpyrazine	1118	a	0.03	0.02	0.03	0.01	
Benzylalcohol (79)	1122	a	0.27a	0.18b	0.19b	0.06	**
Phenylethyl alcohol (91)	1195	a	0.53	0.64	0.81	0.35	
Total			96.59b	103.09ab	115.63a	4.55	*
Total microbial metabolism			605.04b	601.91b	723.03a	24.32	**
Unknown or contaminant compounds							
Carbon disulfide (76)	537	a	5.80	6.42	6.70	1.89	
p-Xylene (91)	892	a	0.09b	0.31a	0.11a	0.09	**
2-Butoxyethanol	953	a	2.45	2.23	2.33	0.33	
4-Methylphenol (108)	1199	a	0.10	0.06	0.08	0.03	
Total			8.45	8.98	9.23	0.55	

Values are reported as means of the two replications within the same treatment, where GSE is grape seed extract added group, CHE the chestnut extract added group and NIT the control group added with sodium nitrite.
Abundance expressed as AU × $10^{\wedge -6}$ (AU: abundance unit, expressed as total ion chromatogram (TIC) or area of the target ion shown in parenthesis).
[a] Linear retention indices (LRI) of the compounds eluted from the GC–MS using a DB-624 capillary column.
[b] Reliability of identification: a, identification by mass-spectrum and by coincidence with the LRI of an authentic standard; b, tentatively identification by mass-spectrum.
[c] Standard error.
[d] P value of natural antioxidant effect □□$p < 0.01$, □$p < 0.05$, different letters in the same row indicate significant differences at $p < 0.05$.

Spice derived VOCs were the major group, due to the use of black pepper. Indeed, limonene, a compound particularly abundant in pepper (Moretti et al., 2004) represented approximately half of the total amount for each treatment. As the same amount of pepper was added to all treatments, differences among the 3 groups might be due to several external factors such as an irregular distribution of the ground pepper in the raw matrix (Montanari et al., 2018), as well as a heterogeneity of the spices themselves caused by different grinding techniques and/or storage times that could have led to differential

oxidative status, odorant losses and sensory attribute changes (Liu et al., 2013; Orav, Stulova, Kailas, & Müürisepp, 2004).

Microbial enzymes degrade free fatty acids through β-oxidation reactions, generating methyl ketones as final products (Flores et al., 2015). Two VOCs belonging to this group were identified, but significant differences were found only for total abundance, which was highest for NIT, the lowest in GSE and CHE samples were similar to both. Since the main microbial populations were not examined in this study, the differences in volatile development due to microbial fermentation cannot be explained directly. However, the role of genus staphylococcus in incomplete β-oxidation are well-known, so the presence of 2-pentanone and 3-octanone were likely related to the presence of these bacteria (Chen, Kong, Han, Xia, & Xu, 2017).

Contrarily to Chen et al., (2017) and Marco et al. (2006), only one ester was observed. However, they worked on fermented sausages manufactured with starter cultures containing staphylococci strains. Staphylococci promote the formation of esters with staphylococci esterase activity being one of the main factors leading to ester formation in dry-fermented sausages (Wang, Li, Yang, Ruan, & Sun, 2016).

Among VOCs generated by bacterial metabolism, products of carbohydrate fermentation form an important group, consisting in 13 identified compounds. Acetaldehyde, 2,3-butanedione and 2,3- butanediol were higher in GSE samples than in CHE and NIT; ethyl alcohol and butanoic acid were lowest in NIT samples, while 3-hydroxy-2-butanone was lowest in CHE samples. VOCs from carbohydrate metabolism were generally the highest for GSE. The great abundance of 2-butanone is remarkable, considering that this compound is known as a by-product LAB metabolism (Montanari et al., 2018), originating from 2,3-butanedione (significantly higher in GSE). Then, 2-butanone is reduced to 2-butanol and 3-hydroxy-2-butanone, that again, was significantly higher in GSE samples and preferentially formed in small diameter sausages (Montanari et al., 2018).

The last group of VOCs related to bacterial metabolism consisted of 19 VOCs from amino acid degradation. 2-methylpropanal and ethylbenzene were higher in CHE samples, while the lowest amounts of 3-methylbutanol and 2-acetyl-1-pyrroline were observed for natural antioxidant products and benzeneacetaldehyde and benzylalcohol were higher in GSE samples than for CHE and NIT. The highest total amino acid

degradation products were observed in NIT samples, followed by CHE and then GSE. This was likely due to 3-methylbutanol, whose content almost doubled in NIT samples while toluene, the most abundant compound, was similar for the three groups. The compounds observed are characteristic of dry-fermented sausages, being reported by several authors (Corral et al., 2013; Marco et al., 2006; Škrlep et al., 2017).

Despite the variability within each group of VOCs, total amounts of microbial metabolites suggest a greater development of microflora in NIT samples that might be related to the antimicrobial activity of phenolic extracts during ripening. Several studies have reported phenolic compounds diffuse into bacterial cells walls and interact with cytoplasmatic proteins, affecting Gram positive bacteria and, particularly, Gram positive cocci (Fasolato et al., 2016; Jayaprakasha et al., 2003; Riazi et al., 2018). It is worth noting that the main populations involved in sausage fermentation processes, LAB and Staphylococci, are both Gram positive bacteria.

The role each compound plays in defining the aromatic profile strongly depends on its abundance and on its perception threshold (Olivares et al., 2015). During the GC-O sessions, 31 aroma notes were perceived by trained assessors (Table 7). Seven aroma compounds were associated with spices, 1 to lipid beta-oxidation, 4 to carbohydrate fermentation, 1 to esterase activity, 11 to amino acid degradation, 4 to lipid auto-oxidation, 2 to unknown compounds (not identified with any of the VOCs identified by GC–MS) and 1 to contaminants. Considering their DF value as an aroma impact index, 11 VOCs had a DF higher than 8. Despite the differences outlined by SPME-GC–MS analysis, panelists did not detect any differences in the olfactometric profile of the three groups. This is likely because all the identified VOCs were observed in the three groups and the main differences among GSE, CHE and NIT samples were attributable only to differences in the single compounds' abundances. As a consequence, GC-O data were displayed combined in a single aromatic profile (Table 7). Most of the identified compounds were previously observed as recurrent in dry-fermented sausages (Flores & Olivares, 2015; Schmidt & Berger, 1998; Söllner & Schieberle, 2009). Amino acid degradation compounds have a key-role in flavor development, contributing with malty, fruity, sweaty flavors and ripened aroma (Chen et al., 2017; Hospital et al., 2015). Indeed, more than half of SPME-GC–MS identified VOCs were also observed in GC-O sessions as odor active compounds in Cinta Senese dry-fermented sausages. They

accounted for one third of the compounds forming the GCO profile and had high DF values. Among them, 2 acetyl-1-pyrroline and methional are considered as the most potent odor active compounds in dry-fermented sausages (Corral, Leitner, Siegmund, & Flores, 2016; Söllner & Schieberle, 2009). Also 3-methylbutanoic acid is considered a potent aroma contributor, giving cheesy, lactic and fatty notes (Flores & Olivares, 2015), while 2,5-dimethylpyrazine is related to meaty and cooked potatoes notes.

The second most represented VOC group were spice-derived, especially α-terpinene, β-myrcene and terpinolene, which were previously reported as odor active compounds (Olivares et al., 2015; Schmidt & Berger, 1998). Another important group was composed of lipid oxidation VOCs, among them hexanal was the most potent odorant (Marco, Navarro, & Flores, 2007; Olivares et al., 2015; Schmidt & Berger, 1998; Söllner & Schieberle, 2009). Indeed, hexanal produces fresh and green notes (Table 7), but it turns to rancid notes as its abundance increases. Lastly, also 2,3-butanedione, derived from carbohydrate fermentation, due to its low threshold (about 4 μg/l) (Hospital et al., 2015), was an important aroma contributor, characterized by buttery-sweet notes and a DF of 10.

Table 7. Odor active compounds identified by GC-O in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	GC-MS[1]		GC-O[2]		RI[3]	Odour description	
	LRI	LRI std	LRI initial	LRI final			DF[4]
Lipid auto-oxidation							
1-Propanol	613	611	613	618	a	Vegetal, green, pungent, fresh, floral,	5
Propanoic acid	814	806	800	810	a	Tasty, fresh, green, cheese, cured, pungent	4
1-Pentanol	827	823	821	827	a	Vegetable, pungent, unpleasant, cabbage, acid	6
Hexanal	841	839	834	844	a	Green, grass, vegetable, fresh	8
Spices							
Linalool	1150	1145	1141	1149	a	Fresh, floral, cabbage, unpleasant, soap	7
β-Terpinene/γ-Terpinene	1158		1160	1167	b	Cooked, cooked vegetable, floral, pungent, resin	8
β-Myrcene	1003	1003	1000	1004	a	Irritating, spicy, pepper, green, leaves, earthy	10
α-Terpinene	1035	1035	1030	1034	a	Mushrooms, wetness, burnt, unpleasant, pungent, pine, woody, earthy	12
Unkown terpene	1075		1076	1080	c	Earthy, green, vegetable, fresh, fruity, cologne	5
Terpinolene	1101	1106	1107	1113	a	Floral, rose, grass, green	11
α-Thujene	934		929	933	c	Sour, vinager, unpleasant, fruity	5
Lipid β-oxidation							
2-Pentanone	735	731	725	733	a	Floral, green, fresh, oxidized fat, cheese	8

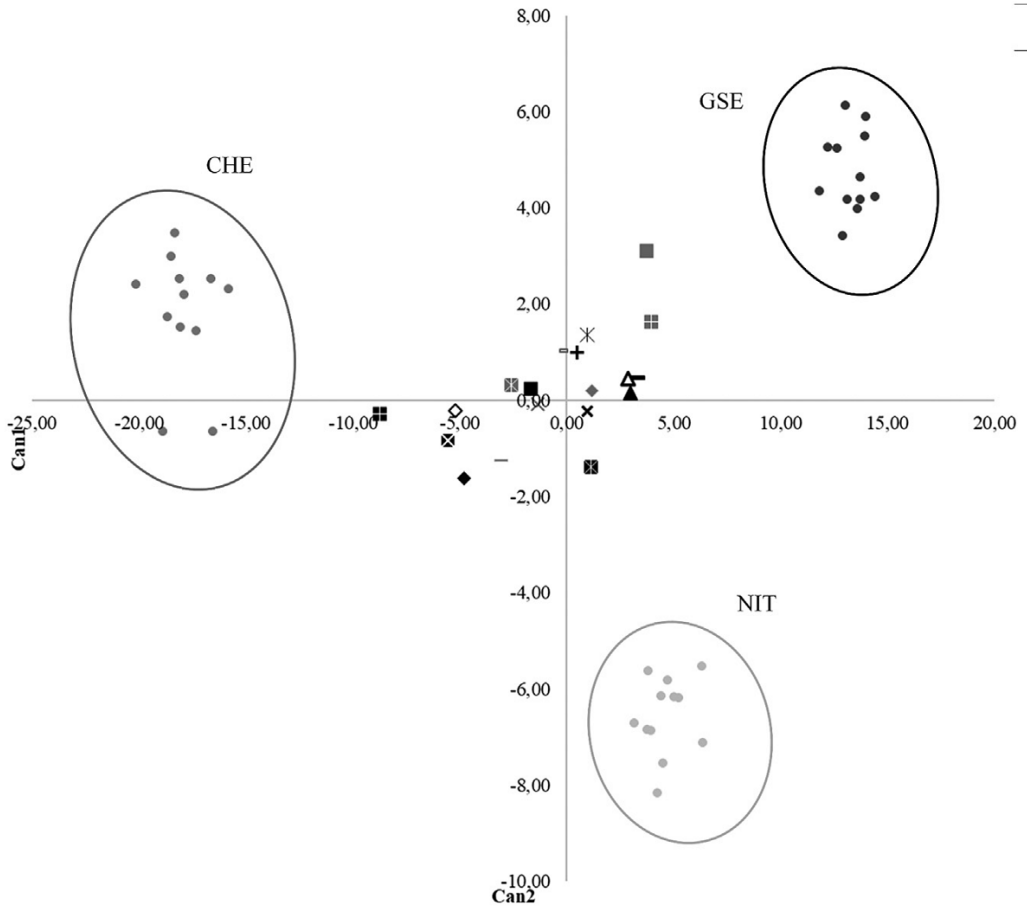
Table 7. Odor active compounds identified by GC-O in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	GC-MS[1]		GC-O[2]		RI[3]	Odour description	
	LRI	LRI std	LRI initial	LRI final			DF[4]
Esterase activity							
3-Methyl-1-butanol acetate	907	905	905	911	a	Sweet, fresh, floral	7
Carbohydrate fermentation							
2-Butanone	632	629	631	642	a	Sweet, slightly unpleasant, green, grass	5
2-Butanol	644	643	650	661	a	Sweet, caramel, malt, unpleasant	5
Acetic acid	720	718	714	722	a	Grass, vegetable, fresh, wine, green	5
Amino acid degradation							
2-Methylpropanal	595	590	602	609	a	Acid, floral, green, weak	4
3-Methylbutanal	691	687	690	697	a	Caramel, chocolate, grass, fresh	6
2-Methylbutanal	702	699	691	700	a	Sweet, floral, fruity, toasted	6
3-Methylbutanol	795	793	791	793	a	Sweet, spicy, toasted, floral	4
2-Methylpropanoic acid	868	864	869	872	a	Cheese, roasted, cured, green, slightly sweet, unpleasant	6
Ethylbenzene	884	881	884	891	a	Earthy, fresh, green, mushroom	4
3-Methylbutanoic acid	942	941	922	926	a	Cheese, rancid, oxidized fat	8
2,5-Dimethylpyrazine	943	943	936	943	a	Meaty, cooked potatoes, sweet, buttery	7
2-Acetyl-1-pyrroline	961	960	960	964	a	Roasted, nuts, bread, pop-corn, biscuits, fried potatoes	12

Table 7. Odor active compounds identified by GC-O in Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

Compound	**GC-MS[1]**		**GC-O[2]**		**RI[3]**	**Odour description**	
	LRI	**LRI std**	**LRI initial**	**LRI final**			**DF[4]**
Methional	986	964	966	969	a	Mashed potato, cooked onion, roasted meat	9
Tetramethylpyrazine	1118	1118	1115	1121	a	Earthy, green, grass, wetness, fresh	7
Unknown or contaminant compounds							
Carbon Disulfide	537	537	531	543	a	Weak, burnt, malt	4
Unknown		776	762	766	c	Cured, meat, acid, fresh, acid	6
Unknown	1190	1182	1176	1182	c	Roasted, fried nuts, biscuits	11

[1] Linear retention index (LRI) of the compounds eluted from the GC-MS and LRI of standard compound.
[2] Initial and end linear retention index of aroma compound in GC-FID-O.
[3] Reliability of identification (RI): (a) identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assessor's descriptors with those described in the Fenaroli's handbook of flavor ingredients (Burdock, 2002); (b) tentatively identification by mass spectrum; (c) unknown compounds.
[4] Detection frequency value.



	Volatile compounds	Origin	Can1	Can2
⊠	2-Pentanone	Lipid β-oxidation	1.12	-1.40
+	Propanoic acid	Lipid auto-oxidation	0.47	0.99
⊠	3-Octanone	Lipid β-oxidation	-5.56	-0.85
▲	Acetaldehyde	Carbohydrate fermentation	2.96	0.15
×	1-Butanol	Carbohydrate fermentation	0.97	-0.23
◆	3-Methyl-butanol	Amino acid degradation	-4.79	-1.63
▬	2-Methyl-butanol	Amino acid degradation	3.27	0.48
■	Pyrrole	Amino acid degradation	-1.67	0.24
◇	Ethylbenzene	Amino acid degradation	-5.23	-0.22
△	2,5-Dimethylpyrazine	Amino acid degradation	2.87	0.45
✳	Benzeneacetaldehyde	Amino acid degradation	0.95	1.37
⊠	Hexane	Lipid auto-oxidation	-2.58	0.31
▦	Octane	Lipid auto-oxidation	3.96	1.63
▫	1-Pentanol	Lipid auto-oxidation	-0.32	1.04
–	Hexanal	Lipid auto-oxidation	-3.05	-1.26
◆	1-Hexanol	Lipid auto-oxidation	1.16	0.19
■	Decane	Lipid auto-oxidation	3.75	3.09
▦	Tetradecane	Lipid auto-oxidation	-8.76	-0.29
×	Tetramethylpyrazine	Amino acid degradation	-1.36	-0.09
	Proportion of explained variation		**0.88**	**0.12**

Fig. 1. Scores of Canonical discriminant analysis for GSE (●), CHE (●) and NIT (●) samples and loadings of Canonical discriminant analysis for volatile compounds identified by Stepwise discriminant analysis.

Figure 1 displays the 19 compounds identified by SDA. The selected compounds were able to discriminate the three treatments. Can1 accounted for a great part of variance, separating CHE from GSE and NIT, while Can 2 sharply divided GSE and CHE from NIT. Multivariate analysis showed how lipid auto-oxidation compounds, comprising half of the compounds identified by SDA, were central in differentiating the three groups. Tetradecane, ethylbenzene and 3-octanone having the greatest negative Can1 scores, were considered mainly responsible for separating GSE and NIT from CHE, in agreement with ANOVA results. Concerning Can2, compounds with higher weighing were decane and octane for GSE and CHE, while 3-methylbutanol seems to characterize NIT samples. Among the 19 compounds identified, 8 were also perceived by GC-O panelists. However, considering their DF and CCs together, only hexanal and 2.5-dimethylpyrazine might have an effective potential in discriminating the groups also from a sensory point of view.

3.3. Sensory analysis

Figure 2 shows sensory results. Abnormal colors, off-flavors, off-odors and rancid were scored as 0 (not present) and were not shown. As expected, the most affected traits were

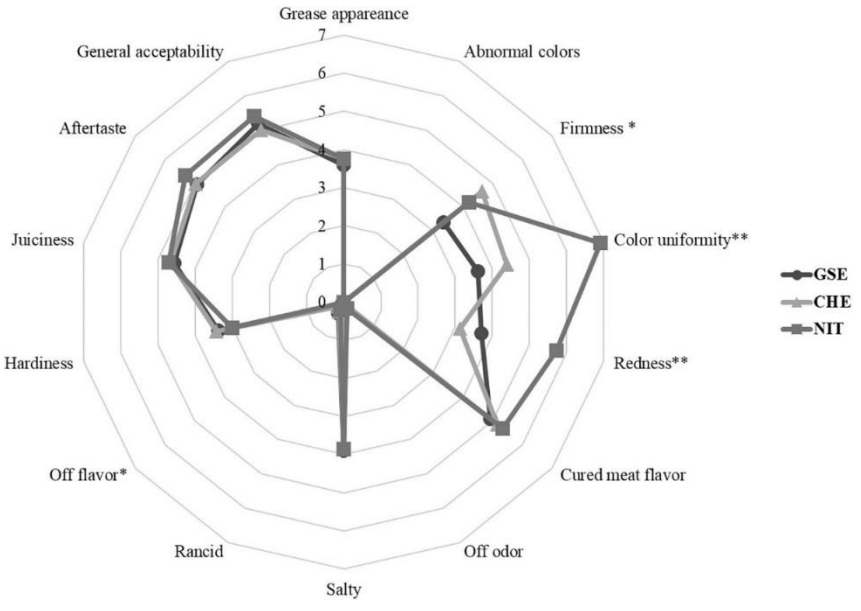


Fig. 2. Sensorial traits of Cinta Sense dry-fermented sausages manufactured with natural antioxidant as replacement of sodium nitrite.

color related, with both color uniformity ($P < 0.01$) and redness ($P < 0.01$) scoring lower for GSE and CHE samples compared to NIT ones, while firmness was scored highest for CHE samples ($P < 0.05$), agreeing with TPA results. Likely, the lower moisture and fat contents observed in CHE could have affected the firmness, even if neither moisture nor fat, significantly differed among treatments.

Despite the differences in lipid auto-oxidation VOCs, no perceivable rancid notes were detected by panelists, as the level of MDA found in the samples did not exceed the organoleptic perception of lipid oxidation (Campo et al., 2006). Effects of adding grape seed and chestnut extracts to dry-fermented sausages have not, however, always been positive. Ribas-Agustí et al. (2014) reported that panelists discarded grape seed extract added products, as there were judged to be abnormal compared to control samples. Similarly, Özvural and Vural (2011) observed a decrease in overall acceptability of frankfurters with grape seed extract added, even when products manufactured with concentrations lower than 0.05% resulted in scores similar to control.

4. Conclusions

The results on VOCs profiles suggested a greater antimicrobial activity of natural antioxidant mixtures (GSE and CHE) compared to sodium nitrite (NIT), likely due to their phenolic constituents; further none of the main foodborne pathogens were found in any sample. No significant differences among treatments were found for lipid oxidation, even if lipid auto-oxidation VOCs suggested a slightly lower antioxidant activity of GSE and CHE compared to sodium nitrite. Despite the differences in single VOCs abundances, the replacement did not affect the overall aroma profile, as outlined by GC-O results and sensory analysis. Some differences in instrumental color and texture negatively affected GSE and CHE products, but the overall acceptability was not influenced. GSE and CHE effects on microbiota in dry-fermented sausages should be studied in depth, however, the results so far indicated that tested antioxidants are valid alternatives to sodium nitrite in Cinta Senese dry-fermented sausages.

Acknowledgments

The research was carried out with funds from the European Union's Horizon 2020 research and innovation program under grant agreement No 634476 (acronym TREASURE). The content of this works reflects only the author's view and the European Union Agency is not responsible for any use that may be made of the information it

contains. We acknowledge Prof. Romani and Phytolab for providing the antioxidant mixtures. Authors also acknowledge the financial support of AGL2015–64673-R (MINEICO, Spain) and FEDER funds.

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OTHER PUBLICATIONS

Annex 1

Non-hypothetical willingness to pay and expected liking analysis of food neophobic consumers towards pork products innovations in five EU countries

Submitted to PLOS ONE:

Authors: Kallas Z., Čandek-Potokar M., Lebret B., Cerjak M., Pugliese C., **Aquilani C.**, Tomažin U., Karolyi, D., Perruchot M.H., Vitale M. & Gil, J.M.

Personal contribution: Italian products manufacturing and collaboration in the execution of the consumer test and results discussion.

Non-hypothetical willingness to pay and expected liking analysis of food neophobic consumers towards pork products innovations in five EU countries

Consumers' non-hypothetical preference and acceptance with Food Neophobia trait

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Abstract

Consumers' personality traits are gaining relevance in understanding consumers' preferences and acceptance for food innovations, in particular, Food Neophobia (FN). The majority of researches used hypothetical surveys to associate FN to consumers' choice with a clear lack of non-hypothetical approaches. On the basis of the expectancy-disconfirmation model, two Non-Hypothetical Discrete Choice Experiments were carried out to investigate FN and consumers' preferences before and after a hedonic evaluation test. The expected liking using the 9-points hedonic scale and the direct numerical probability elicitation were related to FN. Pork health and quality innovations from rustic pig breeds were investigated in Spain, Italy, France, Slovenia and Croatia.

Results showed that the proposed innovations received high expected Purchase Intention (PI) and Willingness to Pay (WTP). The FN personality trait was significantly more associated to the expected WTP, in particular for the unfamiliar innovations. However, after the hedonic evaluation, more negative disconfirmation outcomes appeared with the WTP estimates. The probabilistic elicitation method extracted more variability when associating the expected liking with FN. The difference between the expected and experienced WTP towards the innovations showed higher variability compared to the difference between the expected and experienced PI. Considering the liking experience and the price information in the non-forced choices, the preferences' variability between food neophobic and neophilic towards innovations increased significantly. The longer probabilistic scale that relates the expected liking with FN captured higher variability. It

is worth considering a measurement tool that classifies consumer's perceptions towards food innovations in terms of familiarity for future analysis when associated to FN.

Key words: Hedonic evaluation, Non-Hypothetical Discrete Choice Experiment, Food Innovations, Food Neophobia, Pork Preferences.

1. Introduction

Food health innovations are becoming determinant factors that affect consumers' food choice. Consumers' preference and acceptance of food innovations are multidimensional and rely on a mixture of the product intrinsic and extrinsic cues, expectations and attitudes (Franchi, 2012), socio-economic characteristics (Verbeke, 2005) and personality traits (Spinelli *et al*., 2018), in particular, Food Neophobia (Pliner and Hobden, 1992). Food Neophobia (FN) is an individual-specific and heritable trait (Knaapila *et al*., 2007) that describes human unwillingness to consume unfamiliar food. Although FN has been examined extensively (Lenglet, 2018), the study of the relationship between FN and consumers' food preferences and acceptance in adult samples still limited, in particular in terms of populations studied (Jaeger *et al*., 2017). Furthermore, the majority of FN researches associated this trait to food choice using hypothetical survey frameworks. Accordingly, consumers are asked about their Purchase Intention (PI) and preferred food choice without any consequences on their household budget in which the product price didn't influence their answer. There is a lack in consumers' studies that relate FN to the consumers' non-hypothetical food choice taking into account their budget constraints and product price. Surveys may, in general, suffer from the hypothetical bias which reflects the old saying: "there is a difference between saying and doing" (Loomis, 2014). Letting surveys to be consequential to respondent using non-hypothetical frameworks is an *ex-ante* approach to reduce this bias (Loomis, 2014).

Chen (2007), on the basis of the Ajzen's Theory of Planned Behaviour, analysed in a hypothetical survey the PI of organic food and FN in Taiwan. This study considered only the price variable in an aggregated approach by estimating an interaction term between price and FN without any specific monetary value of the products. Results showed non-significant associations. Hwang, Lee & Lin (2016) analysed the PI with a hedonic evaluation test for health claims using FN as moderator. Results showed a secondary

role of FN in constructing the consumers' final purchase decision. Monteleone *et al*. (2017) in their FN study recognized the importance of price in the consumer purchase decision and stated that consumers may purchase the product they like less due to its lower price. Accordingly, when consumers face a purchase situation, they may choose on the basis of some product cues or information (health, convenience and price…) but "actually prefer the food not chosen". Consumers may trade-off and compromise some products' attribute if the product is cheaper. Therefore, it is relevant to analyse the role of FN with the PI and Willingness to Pay (WTP) towards the unfamiliar food products when the price information is available. Sanjuán-López, *et al*. (2011) analysed the importance of FN in a more realistic PI for local innovative food products, introducing the product price as a relevant attribute but they used a hypothetical experimental approach using the contingent valuation as a unique choice trial scenario.

The FN is also related to food hedonic evaluation. Consumers with high FN tend to exhibit low expected liking for unfamiliar products which are associated to the expected unpleasant taste (Laureati *et al*., 2018). The expected liking, in the majority of researches, is measured using the traditional 9-points hedonic scale by asking consumers to select their liking level from "dislike very strongly" to "like very strongly". However, alternative approaches are available such as ranking, rating or the direct numerical probability elicitation (Lusk, Schroeder & Tonsor 2014). In the latter method, consumers are asked to state their expected liking in a probabilistic way ranging from "0%" where there is no chance that consumers would like the product and 100% where consumers are sure that they would like the product.

In this context, the objective of this study is twofold. Firstly, to associate FN with the consumers' non-hypothetical Purchase Intention and Willingness to PAY using the Non-Hypothetical Discrete Choice Experiment (NH-DCE) method. Innovative pork products from five untapped pig breeds were used in Spain, Italy, Slovenia, Croatia and France. Secondly, to compare the association of the expected liking with the FN trait, using the traditional 9-points hedonic scale and the direct numerical probability elicitation alternative.

This paper contributes to the existing literature of consumers studies in three aspects. Firstly, at the methodological level, we contribute to the very scarce literature that relates FN with non-hypothetical PI and WTP approach using a real purchase environment and

a hedonic evaluation test. For that purpose, we created several non-hypothetical purchase scenarios using a D-optimal choice design by involving real products and money to ensure consequentiality of consumers' decision. We acknowledge that non-hypothetical experiment with real economic incentives were used to relate Food Technology Neophobia (FTN) with consumers' preference (Matin *et al*., 2012; Chen, Anders & An, 2013; Lusk *et al*., 2015). However, studies involving FN in non-hypothetical food choice experiment are not available. Secondly, we contribute to the body of knowledge by comparing two alternatives to elicit expected liking and its association to FN. Thirdly, at empirical level, as recommended by Damsbo-Svendsen *et al*. (2017) we re-tested the reliability of the FN scale across culture and countries. We also make available the used items of FNS in the Catalan, Croatian, French and Slovenian languages.

2. Material and methods

Our theoretical approach relies on the expectancy-disconfirmation model (Oliver, 1980) and in part on the Total Food Quality Model (Grunert *et al*., 1996). It involves a comparison between the cognitive state (expected PI and WTP) prior to an event (hedonic evaluation test) and the subsequent cognitive state (experienced PI and WTP) after the event is experienced. According to this approach, many characteristics of a food product cannot be discovered before purchase. Consumers develop expectations about its quality when make a food choice (Kallas *et al*., 2016) and they rely on its extrinsic attributes to deduce its quality. Once the product is consumed, these expectations may change. If the experience matches the expectation, confirmation occurs, which results in satisfaction. If there is a mismatch, a positive disconfirmation may occur if experience improves expectations and a negative disconfirmation may occur if experience worsens expectations. The experiment was carried out in three-steps:

1. Firstly, in an initial questionnaire, a Non-Hypothetical Discrete Choice Experiment is applied to assess consumers' non-hypothetical "expected" PI and WTP.

2. Secondly, in a further questionnaire, a hedonic evaluation test of the same products analysed in the first step was carried out.

3. Thirdly, the initial questionnaire with the same Non-Hypothetical Discrete Choice Experiment was repeated to assess consumers' non-hypothetical

"experienced" PI and WTP and to analyse the role of the hedonic evaluation in determining the consumers' final decision towards the proposed innovations.

2.1. Consumers sample

Data was collected from a sample of at least 120 consumers in each country (618 consumers in total) having purchased and consumed the pork products proposed in this study during the last month and stratified in terms of gender and age. A quota sampling procedure was used. The experiment was conducted by the authors in: Barcelona (Spain) with 121 consumers during February 2017, Bologna (Italy) with 121 consumers during February 2017, Ljubljana, Maribor and Koper-Capodistria (Slovenia) with 131 consumers during March 2017, Zagreb (Croatia) with 121 consumers during March 2017 and Toulouse (France) with 124 consumers during May 2017. Consumers were motivated and economically compensated to participate (approximately with twenty Euros value in a voucher/gift by respondent). Each experimental session with consumers lasted approximately one hour and half. Table 1 represents a summary of the sample description across countries. The experiment was approved by the ethic committee of the Centre for Agro-food Economy and Development and was conducted according to the ethical principles expressed in the Declaration of Helsinki with a specific care on protecting personal information according to the New European regulations. Before conducting the experiment, the participants signed a consent form and received an explanation of the experiment which was read to them aloud and projected using power point before starting in each case study.

Table 1: Summary of the socio-economic and demographic variables across countries

	Country	**Spain**	**Italy**	**Slovenia**	**Croatia**	**France**
	Sample size	**121**	**121**	**131**	**121**	**124**
Gender	**Female**	48.76%	60.33%	56.49%	49.59%	56.45%
	Male	51.24%	39.67%	43.51%	50.41%	43.55%
Age categories	**18-29 years**	12.40%	38.66%	19.85%	17.36%	11.29%
	30-39 years	21.49%	26.05%	22.90%	23.97%	14.52%
	40-49 years	26.45%	16.81%	22.14%	28.10%	30.65%
	50-59 years	22.31%	10.92%	20.61%	14.88%	20.97%
	>60 years	17.36%	7.56%	14.50%	15.70%	22.58%
Family members	**Average**	2.92	3.23	2.79	3.65	2.41
% with children < 12 years	**Yes**	19.83	18.18	16.79	39.50	16.13

Table 1: Summary of the socio-economic and demographic variables across countries

	Country Sample size	Spain 121	Italy 121	Slovenia 131	Croatia 121	France 124
Number of children < 12 years	**Average**	1.46	1.43	1.23	1.71	1.39
Household perception of the monthly net income compared to the average	**Far below average**	18.18%	0.83%	3.05%	3.31%	7.26%
	Below average	26.45%	14.88%	14.50%	9.92%	20.97%
	Average	32.23%	62.81%	61.07%	49.59%	39.52%
	Above average	18.18%	16.53%	17.56%	32.23%	25.00%
	Far above average	2.48%	0.83%	2.29%	4.13%	3.23%
	I don't know	2.48%	4.13%	1.53%	0.83%	4.03%
Household perception of the monthly food expenditure compared to the average	**Far below average**	5.00%	11.57%	6.11%	3.31%	9.68%
	Below average	21.67%	35.54%	21.37%	19.01%	19.35%
	On average	26.67%	30.58%	41.22%	39.67%	45.16%
	Above average	38.33%	16.53%	26.72%	28.10%	18.55%
	Far above average	5.83%	1.65%	3.05%	8.26%	6.45%
	I don't know	2.50%	4.13%	1.53%	1.65%	0.81%

2.2. Products and innovations

We used several pork products obtained from six untapped and local pig breeds in Croatia (*Turopolje*), France (*Gascon - Noir de Bigorre chain*), Italy (*Cinta Senese*), Slovenia (*Krškopolje*) and Spain (*Porc Negre Mallorquí*). These products fit within the measures that aim to protect the local, autochthonous and untapped pig breeds by creating added-value products that meet consumers' preferences and market demand (EC, 2017). As can be seen in Table 2, different products were selected according to their relevance in each case study market in terms of consumption and our ability to produce the pork products at small scale in enough quantities to be purchased by consumers during the created non-hypothetical purchasing scenarios. The products were patty (Spain), salami (Italy and Slovenia) and dry-cured ham (France and Croatia). These products were produced from the above-mentioned untapped breeds as Traditional Pork Products (TPP). For each identified TPP and case study, we included different innovations targeting quality or healthiness improvement by adding a positive component or reducing a negative one. Several Innovative Traditional Pork Products (ITPP) were identified (Table 2).

Table 2: The traditional and innovative pork products in each case study

Country	Pig breed	Product	CONV	PREM	TPP	ITPP1	ITPP2
Spain	***Negre Mallorquí (NM)***	**Patty**	**Patty Conventional**	**Patty Premium**	**Patty (NM)**	**Patty (NM) & added dietary fibber**	**Patty (NM) & Natural antioxidant**
Italy	***Cinta Senese (CS)***	**Salami**	**Salami Conventional**	**Salami Premium**	**Salami (CS)**	**Salami (CS) & Natural conserving agent**	**-**
Slovenia	***Krškopolje (KRS)***	**Salami**	**Salami Conventional**	**Salami Premium**	**Salami (KRS)**	**Salami (KRS) without nitrites**	**-**
Croatia	***Turopolje (TRP)***	**Dry-cured ham**	**Dry-cured ham Conventional**	**Dry-cured Ham Premium**	**Dry-cured ham (TRP)**	**Dry-cured ham (TRP) less salting time**	**Dry-cured ham (TRP) less smoking time**
France	***Gascon - Noir de Bigorre chain (NB)***	**Dry cured ham**	**-**	**Dry-cured ham 50% Iberian**	**Dry-cured ham (NB) 24 months ripening**	**Dry cured ham (NB) 36 months ripening**	**-**

The ITPP in Spain was obtained by enriching the patties with Porcini (*Boletus edulis*) as a natural source of dietary fibre (*Beta glucans*) and Blueberries (*Vaccinium corymbosum*) as a natural source of antioxidants. In Italy, the ITPP salami was produced with natural preserving agent. In Slovenia the ITPP salami was produced without nitrites. In Croatia the ITPP dry-cured ham was produced with reduced salting time and with less smoking. In France the ITPP dry-cured ham was produced by increasing the ripening time from 24 to 36 months to enhance the eating quality; especially, improvement of aroma and flavour was foreseen as a result of increased lipolysis and proteolysis processes with ripening time.

The main criteria used in the election of each innovation within each case study were: a) the relevance of the innovation in tackling with the most relevant consumers' health concerns. The proposed innovations may contribute to diseases prevention related to salt consumption such as the hypertension (Campbell *et al*., 2011), preventing cardiovascular

diseases related to the consumption of natural antioxidant or reducing the gastro-intestinal diseases related to nitrites additives consumption (Knekt, *et al*., 1999). b) Our capacity to include the innovations and produce the ITPP at small scale for the experiment performance, c) Our ability to afford the production cost due to budget constraints and d) the availability of meat or products taking into account the limited resources of the untapped breeds according to each case study.

The TPPs and the ITPPs produced from the untapped breeds were compared with two additional products obtained from commercial pig breeds. The first product was with "conventional quality" (CONV) that met the standards and the minimum requirements of the production process with relatively "normal" or low prices. The second product was with "premium quality" (PREM) that goes beyond the minimum standard and quality requirement with relatively higher prices. Both the CONV and the PREM products were produced in each case study to ensure homogeneity in the production qualities when compared to the TPP and the ITPP. Finally, in the French case study, the CONV option was not introduced because the TPP from the untapped breed is already available at market place with a clear differentiation in term of perceived quality and price from conventional products. Thus, in this case, only the PREM was defined. The CONV, PREM, TPP and ITPP from the five case studies were used for the assessment of the consumers' non-hypothetical PI and WTP.

Table 3 Price vectors of the products by countries

	Spain	**Italy**	**Slovenia**	**Croatia**	**France**
Products	Patties 250 g Tray of 2 patties	Salami 100 g Vacuum sliced	Salami 200 g Vacuum one piece	Dry-cured ham 100 g Vacuum sliced	Dry-cured ham 100 g Vacuum sliced
TPP	3.00€,3.75€ 4.50€,5.25€	1.80€,2.00€ 2.20€,2.40€	3.60€,4.00€ 4.40€,4.80€	11.00Kn,12.00Kn 13.00Kn,14.00Kn	12.80€,14.40€ 16.00€,17.60€
ITPP1	3.00€,3.75€ 4.50€,5.25€	1.80€,2.00€ 2.20€,2.40€	3.60€,4.00€ 4.40€4.80€	11.00Kn,12.00Kn 13.00Kn,14.00Kn	17.60€,20.00€ 22.40€,24.80€
ITPP2	3.00€,3.75€ 4.50€,5.25€	-	-	11.00Kn,12.00Kn 13.00Kn,14.00Kn	-

Table 3 Price vectors of the products by countries

	Spain	Italy	Slovenia	Croatia	France
CONV	2.00€,2.50€ 3.00€,3.50€	1.20€,1.40€ 1.60€,1.80€	2.40€,2.80€ 3.20€,3.60€	8.00Kn,9.00Kn 10.00Kn,11.00Kn	-
PREM	3.00€,3.75€ 4.50€,5.25€	1.60€,1.80€ 2.00€,2.20€	3.20€,3.60€ 4.00€,4.40€	10.00Kn,11.00Kn 12.00Kn,13.00Kn	8.00€,9.60€ 11.20€,12.80€

2.3. Analysing the non-hypothetical PI and WTP for pork innovative products

We used the Non-Hypothetical Discrete Choice Experiment (NH-DCE) methodology to analyse consumers' PI and WTP, measured before and after a hedonic evaluation test. The previously defined pork products (TPP, ITPP CONV, and PREM) were jointly presented to respondents in an array of repeated simulated purchase situations (cards) at different price levels. The "NONE" option (i.e. neither of them) was also included to be consistent with the demand theory and to make the choice task more realistic as this option is available when shopping. Respondents were asked to select the product that they would purchase for sure in a simulated market situation, thereby revealing their preference for certain characteristics of the products. Each product type was assigned four price levels. The products format and price levels are presented in Table 3.

We defined eight purchase situations (Supplementary Data 1) using a D-optimal fractional factorial choice design (Lusk & Shroeder, 2004) the Ngene software (ChoiceMetrics, 2016). To ensure the non-hypothetical nature of the experiment, before the NH-DCE questions, participants were informed that one product will be delivered from a randomly selected purchase situation. Thus, consumers were rewarded by "extra money" that covered the highest price level of the products plus an additional margin ranging between 10% and 30% depending on the product and the budget in each case study. Consumers were also informed that all the products are "real" one and produced to be "sold" at the end of the experiment. The non-hypothetical nature of the experiment implies an interchange of money and preferred products. Firstly, we randomly select which NH-DCE is binding (i.e. before or after the hedonic evaluation) by having one of the participants draw a number out of an envelope from 1 to 2. Secondly, we randomly select which choice situation is binding by having one of the participants draw a number

out of an envelope from 1 to 8. Once the randomly purchase situation is identified, consumers were requested to look for their answers. If the NONE option was selected no product is delivered and consumers were invited to leave the experiment room. If consumers selected any other product, they were asked to pay its posted price and to take their selected product.

The DCE relies on Lancaster's Theory of Value (Lancaster, 1966) and on the Random Utility Theory (RUT) of Thurstone (1927). The individuals choose among the product, in a purchase situation, according to a utility function with two main components: a systematic (observable) component and a random error term (non-observable):

$$U_{jn} = V_{jn} + \varepsilon_{jn} \quad (1)$$

where U_{jn} is the utility of product j to subject n, V_{jn} is the systematic component of the utility and ε_{jn} is a stochastic term. In our case, the utility function for product j can be expressed as:

$$V_{jn} = \beta_j . ASC_j + \sum_{k=1}^{J} \alpha_{jk} . P_{kn} \quad (2)$$

Where j are the TPP, ITPP1, ITPP2, CONV, and PREM products. P_{kn} is the kth product's price for consumer n, β_j are the coefficients of the Alternative Specific Constant (ASC_j) for each product *j* which represents the marginal utility of the product *j*. α_{jk} are the coefficients representing the effect of the j^{th} product price on the utility for the j^{th} product.

To predict the subjects' choice for a product, we used the Random Parameters Logit (RPL) model. In this case, the coefficient vector of the ASC is decomposed as $\beta_j = \bar{\beta} + \sigma \lambda_n$, where $\bar{\beta}$ is the estimated mean of the ASC and σ is the standard deviation of the marginal distribution of $\bar{\beta}$ and λ_n is a random term assumed normally distributed with mean zero and unit standard deviation. The price coefficients are considered as fixed parameters to ensure that the estimated total WTP is normally distributed. The WTP of a product j versus the baseline alternative NONE is

calculated as the negative ratio of the ASC coefficient to the price coefficient of the same product *j* (Lusk and Schroeder, 2004):

$$WTP_{\text{Product j Vs. No-option}} = -\left(\frac{\frac{d}{dACS_j}\beta_j.ASC_j}{\frac{d}{dP_{kn}}\alpha_{jk}.P_{kn}} \right) = -\left(\frac{\beta_{\text{j}}}{\alpha_{\text{jk}}} \right) = \left(\frac{\beta_{\text{Product j}}}{\alpha_{\text{price j}}} \right) \quad (3)$$

Finally, the Krinsky and Robb parametric bootstrapping method was applied to calculate the confidence intervals of the WTPs with 1,000 random repetitions using NLOGIT 5.0 software.

2.4. Measuring the FN

Recently, Damsbo-Svendsen *et al*. (2017) carried out a review in which they reported thirteen instruments to measure FN. While they did not identify a superior measurement tool, they mentioned that the Food Neophobia Scale (FNS) developed by Pliner and Hobden (1992) is currently the most widely used psychometric tool to investigate FN and to predict consumers' response towards new food products (Spinelli *et al*., 2018; Ritchey *et al*., 2003). This scale consists of five positive and five negative statements towards different situations of food consumption, using 9-points Likert scale with the following categories: "disagree very strongly", "disagree strongly", "disagree moderately", "disagree slightly", "neutral: do not agree nor disagree", "agree slightly", "agree moderately", "agree strongly" and "agree very strongly". The original scale version was translated to the different languages and tested for the comprehension of the items in a pilot sample of about 10-16 consumers in each country. Some items and words have been adapted and improved to ensure comprehension. The used versions of the FNS in each case study are provided in Supplementary Data 2. The internal consistency and validity of the scale was measured by the Cronbach-Alpha and the Principal Component Analysis (PCA). The individual FN score was calculated by summing all the ratings of positive statements with reversed scores of negative statements.

To assess the FN association with the non-hypothetical PI and WTP, we carried out Two-Step Cluster Analysis (TSCA) technique rather than splitting the FN scores into tertiles as proposed by Fernández *et al*. (2013). The classification procedure was based on the

Log-likelihood measure that places a probability distribution on the FNS score to identify the optimum number of the cluster by identifying the corner change in the values and thus the "natural" grouping of consumers. The Silhouette coefficient was used to test the cluster quality extraction.

2.5. Measuring consumers' expected liking: The Hedonic evaluation test

The hedonic evaluation test of the TPP, ITPP, CONV and PREM was carried out following the procedure described in Napolitano *et al*. (2007). The 9-points hedonic scale from "I extremely dislike" to "I extremely like" was used. The sensory experience was conducted in three steps, separated by a 5-10 minutes' break (Sanjuán-López, *et al*. 2011) using individual and separated questionnaire sheet in each part (Napolitano *et al*., 2010). The consumers were offered the TPP, the ITPP, the CONV and the PREM products in random order (Cerjak *et al*., 2011). Firstly, they were asked to taste the products in a blind condition with no additional information (blind liking). After that, the respondents received a sheet that contained the description of the products in exactly the same way to the choice purchase situation but without any price information. They were asked to carefully read the information and to state their liking scores (expected liking). In the third step, consumers were given the products to taste with an information sheet that allowed them to identify which specific product they are tasting (actual liking).

3. Results

3.1 The non-hypothetical WTP and PI for food innovations in pork products

Two RPL models were estimated before and after the hedonic evaluation test (Table 4). Results showed that at 99% confidence level, we can reject the null hypothesis that all coefficients are jointly equal to zero with a highly acceptable goodness of fit (McFadden's pseudo-R^2). The positive/negative sign of the coefficients implies higher/lower levels of utility associated with the products, and thereby with their characteristics. The model estimates showed that almost all coefficients are statistically significant in all countries and between treatments. All the estimated standard deviations of the random coefficients (ASCs) were highly significant, confirming the presence of non-observed heterogeneity and thus the suitability of the model. However, estimates cannot be compared between treatments due to the scale parameter. Comparisons can only be evaluated at the WTP level using the Poe test (Poe *et al*., 2005).

Following equation 3, we estimated the expected and experienced WTPs. Results (Table 5) showed a positive expected preference, in general, of the new products proposed from the untapped pig breeds and the innovations. The expected WTP showed the highest values for the TPP and the ITPP compared to CONV and PREM products in all countries with the exception of Spain were the expected WTP for the ITPP1 (patties enriched with natural source of dietary fibber) was similar to the CONV product and the expected WTP for the ITPP2 (patties enriched with natural antioxidant) was similar to the PREM product.

After the hedonic evaluation, the expected WTP for the TPP were confirmed (C) by the experienced WTP in all countries where non-significant differences were identified. The hedonic evaluation, in this case, had no significant impact on consumers' WTP. For the ITPP, results showed that after the hedonic evaluation the expected WTP was negatively disconfirmed (ND) (decreased significantly) for the majority of innovations, in all countries, with the exception of the ITPP1 in Spain whose expected WTP was confirmed to the experienced one (C). Consumers expected more from the proposed innovations in terms of taste and therefore the hedonic evaluation played a relevant role in determining the final preference patterns.

Table 4: Random Parameters Logit models before and after the hedonic evaluation test

β_s	Spain		Italy		Slovenia		Croatia		France	
	RPL		*RPL*		*RPL*		*RPL*		*RPL*	
	Expected	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**
Random β_s										
ASC-TPP β_1	**4.77*****	**6.40*****	**5.84*****	**14.34*****	**4.96*****	**11.42*****	**11.86*****	**9.72*****	**11.92*****	**10.86*****
ASC-ITPP1 β_2	**4.00*****	**3.25*****	**8.95*****	**7.78*****	**11.50*****	**11.33*****	**12.67*****	**13.89*****	**8.13*****	**5.07****
ASC-ITPP2 β_3	**4.64*****	**2.06*****					**5.30****	**-0.76**		
ASC-CONV β_4	**3.06*****	**2.63*****	**-1.72**	**2.78*****	**0.92**	**4.22*****	**1.23**	**1.89**		
ASC-PREM β_5	**4.95*****	**3.29*****	**4.02*****	**10.06*****	**5.30*****	**12.23*****	**4.85*****	**3.01**	**3.17*****	**4.32*****
Non-random α_s										
PRICE-TPP α_1	**-1.36*****	**-1.77*****	**-2.19*****	**-6.73*****	**-1.13*****	**-3.13*****	**-0.78*****	**-0.62*****	**-0.78*****	**-0.73*****
PRICE-ITPP1 α_2	**-1.27*****	**-1.25*****	**-3.74*****	**-3.49*****	**-2.34*****	**-3.73*****	**-0.88*****	**-1.11*****	**-0.54*****	**-0.48*****
PRICE-ITPP2 α_3	**-1.28*****	**-1.19*****					**-0.36*****	**-0.64*****		
PRICE-CONV α_4	**-1.12*****	**-1.01*****	**-2.33*****	**-3.58*****	**-1.46*****	**-2.42*****	**-0.61*****	**-0.56*****		
PRICE-PREM α_5	**-1.38*****	**-1.22*****	**-2.88*****	**-8.11*****	**-2.06*****	**-3.32*****	**-0.50*****	**-0.43*****	**-0.53*****	**-0.73*****
S.D. σ_s of random β_s										
S.D. TPP σ_1	**3.31*****	**5.13*****	**2.29*****	**4.57*****	**2.40*****	**6.13*****	**2.94*****	**7.29*****	**4.07*****	**4.68*****
S.D. ITPP1 σ_2	**2.43*****	**3.48*****	**2.71*****	**5.16*****	**2.77*****	**7.33*****	**4.43*****	**6.85*****	**5.32*****	**8.89*****
S.D. ITPP2 σ_3	**2.87*****	**5.68*****					**4.06*****	**17.1*****		
S.D. CONV σ_4	**2.74*****	**3.95*****	**4.38*****	**4.67*****	**2.34*****	**3.90*****	**3.45*****	**6.51*****		

Table 4: Random Parameters Logit models before and after the hedonic evaluation test

β_s	Spain		Italy		Slovenia		Croatia		France	
	RPL		*RPL*		*RPL*		*RPL*		*RPL*	
	Expected	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**	**Expected**	**Experienced**
S.D. PREMσ_5	**3.52*****	**5.19*****	**2.88*****	**8.52*****	**3.26****	**6.41*****	**3.92*****	**4.67*****	**3.70*****	**5.24*****
Pseudo R^2	**0.33**	**0.45**	**0.38**	**0.52**	**0.41**	**0.52**	**0.50**	**0.60**	**0.42**	**0.49**

Table 5: Willingness to Pay (WTP) and Purcahse Intention (PI) before and after the hedonic evaluation test

Products	**Spain**		**Italy**		**Slovenia**		**Croatia**		**France**	
	Expect.	***Exper.***	***Expect.***	***Exper.***	***Expect.***	***Exper.***	***Expect.***	***Exper.***	***Expect.***	***Exper.***
TPP (PI%)	**14.6%**^{y}	**21.8%**^{x}	**35.2%**^{x}	**26.5%**^{y}	**22.1%**^{x}	**20.7%**^{x}	**30.4%**^{x}	**30.3%**^{x}	**34.7%**^{x}	**33.2%**^{x}
ANOVA	**PD**		**ND**		**C**		**C**		**C**	
TPP (WTP)	**3.48€**^{***a}	**3.60€**^{***a}	**2.66€**^{***a}	**2.13€**^{***a}	**4.35€**^{***a}	**3.63€**^{***a}	**15.17**kn^{***a}	**15.58**kn^{***a}	**15.21€**^{***a}	**14.70€**^{***a}
Poe test	**C**		**C**		**C**		**C**		**C**	
ITPP1 (PI %)	**10.8%**^{x}	**10.5%**^{x}	**34.3%**^{x}	**34.2%**^{x}	**48.7%**^{x}	**17.9%**^{y}	**30.1%**^{x}	**22.2%**^{y}	**15.7%**^{x}	**15.3%**^{x}
ANOVA	**C**		**C**		**ND**		**ND**		**C**	
ITPP1 (WTP)	**3.13€**^{***b}	**2.59€**^{***b}	**2.39€**^{***a}	**2.22€**^{***a}	**4.90€**^{***a}	**3.03€**^{***a}	**14.38**kn^{***a}	**12.44**kn^{***b}	**15.02€**^{***a}	**10.47€**^{***b}
Poe test	**C**		**ND**		**ND**		**ND**		**ND**	
ITPP2 (PI %)	**18.7%**^{x}	**18.6%**^{x}	-	-	-	-	**23.5%**^{x}	**18.0%**^{y}	-	-
ANOVA	**C**		-		-		**ND**		-	
ITPP2 (WTP)	**3.60€**^{***a}	**1.73€**^{**b}	-	-	-	-	**14.45**kn^{***a}	**-1.18**kn^{d}	-	-
Poe test	**ND**		-		-		**ND**		-	
CONV (PI %)	**24.6%**^{x}	**21.8%**^{x}	**6.3%**^{y}	**12.5%**^{x}	**2.5%**^{y}	**11.4%**^{x}	**2.4%**^{y}	**12.0%**^{x}	-	-

Table 5: Willingness to Pay (WTP) and Purcahse Intention (PI) before and after the hedonic evaluation test

Products	Spain		Italy		Slovenia		Croatia		France	
	Expect.	*Exper.*	*Expect.*	*Exper.*	*Expect.*	*Exper.*	*Expect.*	*Exper.*	*Expect.*	*Exper.*
ANOVA	**C**		**PD**		**PD**		**PD**		**-**	
CONV (WTP)	**2.72€^{***b}**	**2.60€^{***b}**	**-0.73€^{c}**	**0.77€^{***b}**	**0.63€^{c}**	**1.74€^{***b}**	**2.00**kn^{c}	**3.36**kn^{d}	-	-
Poe test	**C**		**PD**		**PD**		**PD**		**-**	
PREM (PI %)	**19.3%^{x}**	**14.9%^{y}**	**11.2%^{x}**	**13.6%^{x}**	**9.9%^{y}**	**30.2%^{x}**	**9.1%^{x}**	**10.3%^{x}**	**16.0%^{x}**	**18.9%^{x}**
ANOVA	**ND**		**C**		**PD**		**C**		**C**	
PREM (WTP)	**3.57€^{***a}**	**2.69€^{***b}**	**1.39€^{***b}**	**1.24€^{***b}**	**2.56€^{***b}**	**3.67€^{***a}**	**9.67**kn^{***b}	**6.95**kn^{***c}	**5.93€^{***b}**	**5.90€^{***c}**
Poe test	**ND**		**C**		**PD**		**C**		**C**	
NONE (%)	**12.0%^{x}**	**12.4%^{x}**	**13.0%^{x}**	**13.1%^{x}**	**16.8%^{x}**	**19.8%^{x}**	**4.6%^{y}**	**7.2%^{x}**	**33.6%^{x}**	**32.7%^{x}**
ANOVA	**C**		**C**		**C**		**PD**		**C**	

PI: Purchase intention (%). WTP: Willingness to Pay in € (Euros) and Kn (Croatian Kuna)
PD: Positive Disconfirmation, ND Negative Disconfirmation, C: Confirmation (Poe test and ANOVA)
Within each case-study, products with different superscript letters in rows (x,y) differ ($P < 0.05$).
a, b, c, refer to the difference across products by column at 95% confidence interval.
***P<0.01.
Expect.: Expected (i.e. before the hedonic evaluation).
Exper.: Experienced (i.e. after the hedonic evaluation).
Shadowed cells show results divergence of the impact of the hedonic evaluation on the WTP and PI.

The PI in Table 5 was also calculated by summing, for each product, how many times were chosen as the most preferred product in all choice sets, independently of its price. Analysing the expected PI, results showed a relatively high rate of preference for the TPPs and the ITPPs compared to the CONV, PREM and NONE alternatives in all countries, except Spain. This difference of the product in Spain compared to the other case studies could be related to the product type used (patty) with fresh meat and most likely with lower perceived value for consumers compared to other products. The highest percentage of PI was found in Croatia, followed by Slovenia, Italy, France and finally in Spain. The estimated share of the products from the untapped breeds shows a potential high consumers' preference at market level. In Spain and France, the expected PI for the TPP jointly with the ITPPs were relatively the lowest (44.10% and 50.40% respectively). Average expected PI were found in Slovenia and Italy (70.8% and 69.50%). Consumers in Croatia showed the highest expected PI (84.00%).

The impact of the hedonic evaluation on the expected PI was heterogeneous. The expected PI for the TPP increased in Spain (positive disconfirmation, PD), but decreased in Italy (negative disconfirmation, ND), and remained unchanged in Slovenia, France and Croatia (confirmation, C). The results of the ITPPs showed that the hedonic evaluation negatively affected the expected PI in Slovenia and Croatia (negative disconfirmation, ND) and remained unaffected in Spain, Italy and France (confirmation, C).

Comparing the impact of the hedonic evaluation on the WTP and the PI, results (Table 5) showed some foreseeable divergence (the shadowed cells in Table 5). The product with the highest PI does not necessary imply the highest WTP (Lusk and Schroeder, 2014). This outcome shows the importance of considering the product own price and that of their competing counterparts when analysing preferences and understanding consumers' reaction to food innovations.

3.2 The Food Neophobia trait

The internal consistency of the scale (Cronbach-Alpha) in Spain was 0.847, in Italy 0.781, in Slovenia 0.877, in Croatia 0.755 and in France 0.710. All values demonstrate highly acceptable validity level. Before estimating the FN score of each respondent, we first checked for the factor structure of the FNS using Principal Component Analysis

(PCA). Two factors were identified. The low food neophobic factor (Low FN F1) and the high food neophobic factor (High FN F2). In general, the PCA correctly separated the positive items from the negative ones. Thereby, confirming the suitability of the FNS to describe the FN trait. However, not all items were classified as expected within the PCA. The item 9 (I would eat almost anything) showed an expected factor load only in Italy, Slovenia and France and the item 8 (I am very particular about foods I eat) was not well associated to the expected factor in the case of Croatia and France. The PCA results carried out on the pooled dataset (all countries) also confirmed the unexpected loads of the items 8 and 9. Therefore, we discarded the items 8 and 9 for the further analysis in order to allow comparison across countries. A new PCA was estimated whose results are presented in Table 6. The goodness of fit and the consistency of this PCA significantly

Table 6: The PCA analysis and Individual FNS score after dropping the items 8 and 9

	Spain		**Italy**		**Slovenia**		**Croatia**		**France**	
The FNS items	**F1**	**F2**	**F1**	**F2**	**F2**	**F1**	**F1**	**F2**	**F1**	**F2**
Item 1	**0.76**	-0.20	**0.49**	-0.44	**0.80**	-0.09	**0.80**	-0.07	**0.74**	-0.14
Item 4	**0.88**	-0.18	**0.79**	-0.22	**0.81**	-0.30	**0.74**	-0.27	**0.67**	-0.26
Item 6	**0.75**	-0.15	**0.68**	-0.43	**0.72**	-0.27	**0.74**	-0.05	**0.73**	-0.11
Item 10	**0.86**	-0.21	**0.84**	-0.24	**0.58**	-0.56	**0.75**	0.16	**0.65**	-0.01
Item 2	-0.28	**0.77**	-0.14	**0.71**	-0.22	**0.76**	-0.10	**0.68**	-0.13	**0.77**
Item 3	0.04	**0.78**	0.05	**0.80**	-0.19	**0.85**	-0.14	**0.75**	-0.10	**0.82**
Item 5	-0.47	**0.59**	**-0.60**	-0.16	-0.29	**0.77**	-0.01	**0.64**	-0.08	**0.75**
Item 7	-030	**0.73**	-0.40	**0.66**	-0.21	**0.82**	-0.31	**0.75**	-0.23	**0.79**
Explained variance (%)	**38.4**	**27.6**	**32.2**	**26.2**	**29.8**	**39.6**	**30.2**	**26.3**	**25.7**	**32.0**
Total Explained variance (%)	**66.0**		**58.40**		**68.46**		**56.46**		**57.69**	
KMO Test	**0.794**		**0.777**		**0.868**		**0.752**		**0.739**	
Bartlett Test (significance)	**441.6 (0.000)**		**318.2 (0.000)**		**498.0 (0.000)**		**246.4 (0.000)**		**287.2 (0.000)**	
NFS score	**27.68^{x}**		**$26.64^{x,y}$**		**25.07^{y}**		**$26.93^{x,y}$**		**22.37^{z}**	
Std. Deviation	10.56		9.60		10.11		11.05		8.49	
Min.	9.00		10.00		8.00		8.00		8.00	
Max.	59.00		49.00		65.00		72.00		40.0	

FNS scores across countries with different superscript letters in rows (^{x,y,z}) differ ($P < 0.05$)

increased after dropping the items 8 and 9.

For the estimation of consumers FN, the individual FNS score was calculated by summing all the ratings of positive statements with reversed scores of negative statements. Results (Table 7) showed that consumers in France were the lowest food neophobic. The Spanish consumers exhibited higher FN compared to the Slovenian one, while Italian and Croatian consumers shared their FN with the average scores. Results of the Two-Step Cluster Analysis showed three natural clustering structures in all countries (Table 7): The Low neophobic cluster (Low FN C1), the neutral neophobic cluster (Average FN C2) and the high neophobic cluster (High FN C3). In all countries, the average silhouette measure of cohesion and separation showed a good cluster quality with 0.7 values in all case studies.

Table 7: Results of the Two Steps Cluster Analyses using the adapted NFS.

	Country	Spain	Italy	Slovenia	Croatia	France
Cluster 1 Low FN C1	**Size (%)**	**20.0%**	**38.8%**	**42.3%**	**26.7%**	**54.0%**
	Consumers number	**24**	**47**	**55**	**32**	**67**
	FNS score	**13.67**	**17.30**	**15.65**	**13.84**	**16.03**
	Standard deviation	**2.47**	**2.88**	**3.73**	**2.86**	**4.03**
Cluster 2 Average FN C2	**Size (%)**	**34.2%**	**38.0%**	**34.6%**	**30.0%**	**31.5%**
	Consumers number	**41**	**46**	**45**	**23.57**	**39**
	FNS score	**23.16**	**27.57**	**26.96**	**23.57**	**26.31**
	Standard deviation	**2.95**	**3.11**	**3.05**	**2.62**	**3.00**
Cluster 3 High FN C3	**Size (%)**	**45.8%**	**23.1%**	**23.1%**	**43.3%**	**14.5%**
	Consumers number	**55**	**28**	**30**	**52**	**18**
	FNS score	**36.60**	**40.79**	**38.17**	**36.43**	**37.44**
	Standard deviation	**6.06**	**4.29**	**3.45**	**5.50**	**1.94**

3.2.1 FN association with the non-hypothetical WTP and PI

The association of consumers' FN and the non-hypothetical WTP and PI are presented in Table 8. Comparisons were carried out between only low and high FN clusters to better highlight the FN role. Focusing on the TPP, low FN consumers showed higher non-hypothetical PI compared to the high FN ones for almost all countries and treatment. However, there was a significant change for the expected PI in Italy (40.69% compared to 21.43%). These findings are likely associated to the nature of the products TPP. In fact, all the proposed products can be considered as familiar one (patty, salami and dry-cured ham). Furthermore, in all countries, low and high FN consumers showed similar non-hypothetical PI for the CONV and the PREM products.

Regarding the innovations (ITPPs), Analysing the expected PI, a clear tendency was identified where low FN consumers exhibited higher percentages than the high FN ones. Food neophobic consumers showed some reluctance regarding the innovative pork products. However, significant differences were only found in Spain. A remarkable pattern can be identified. The association of the FN trait with the expected PI was not significant when the innovation consisted of an elimination or a reduction of relatively known component in a familiar product to improve consumers' health (dry-cured ham with less salt, dry-cured ham with less smoke, salami without nitrites). However, when innovation consisted of adding new and unfamiliar components such as those introduced in the ITPPs in Spain, results showed highly significant difference between low and high FN consumers.

In Spain, low FN consumers showed the highest expected and experienced PI for ITPP and the TPP compared to the high FN consumers. However, significant differences were only found for the expected and experienced PI of the ITPP2 (31.77% compared to 14.32% and 27.60% compared to 16.76% respectively) and for the expected PI of the ITPP1 (12.50% compared to 7.27%). In Italy, similar results were found. The FN was not associated to the expected PI for the ITPP but to the experienced PI. Low FN consumers showed higher experienced PI (50.53%) than the high FN one (22.32%). In Slovenia and Croatia, results showed a clear tendency in which consumers with high FN, compared to the low FN, were reluctant to select the TPP and the ITPP both before and after the hedonic evaluation. However, non-significant differences were found. In France, the low FN consumers tended to select more the ITPP. However, non-significant difference was found for the expected PI.

An additional finding is related to changes occurred to the expected PI after the hedonic evaluation test and their relation to the FN. The hedonic evaluation accentuated the appearance of a significant difference of the experienced PI between the low FN and the high FN consumers in Italy and France. Furthermore, the percentage of times that the NONE option was selected turned to be significantly higher for the high FN consumers. Results showed that, in the proposed purchase situations, consumers with high FN tended to select more the option "neither of them" than the low FN ones. This relation was also accentuated after the hedonic evaluation in Italy and Slovenia.

Table 8: Food Neophobia and the non-hypothetical WTP and PI

	Products	Spain		Italy		Slovenia		Croatia		France	
		Expec.	Exper.	Expec.	Exper.	Expec.	Exper.	Expec.	Exper.	Expec.	Exper.
Low FN size		24 consumers		28 consumers		55 consumers		32 consumers		67 consumers	
Purchase intention (%)	TPP	17.71	26.56	40.69[a]	21.01	19.32	22.95	25.78	30.28	36.75	33.02
	ITPP1	12.50 [a]	12.50	35.11	50.53[a]	55.91	18.86	33.20	29.08	13.06	15.80[a]
	ITPP2	31.77[a]	27.60[a]	-	-	-	-	24.61	14.34	-	-
	CONV	18.75	20.83	4.26	10.10	1.14	14.77	10.94	15.94	-	-
	PREM	15.63	8.85	7.45	7.18	9.32	28.40	0.39	5.18	18.47	19.70
	NONE	3.65[b]	4.10[b]	12.50	11.17[b]	14.32	15.00[b]	5.08	5.18	31.72	31.34
WTP (€/product)	TPP	3.87 [a]	4.31	2.78 [a]	2.08	4.29	3.68	15.04	15.34	15.30	14.77
	ITPP1	3.60 [a]	2.70	2.40	2.78 [a]	5.15	3.23	14.64	13.14	14.49	9.24 [a]
	ITPP2	4.60 [a]	2.34	-0.73	0.76	0.52	1.94	15.71	-4.42		
	CONV	2.71	2.90	1.26	1.08	2.64	3.60	1.57	2.68		
	PREM	3.52	2.77	2.78	2.08	4.29	3.68	9.80	8.51	6.54	6.48
Average FN size		41 consumers		46 consumers		45 consumers		36 consumers		39 consumers	
Purchase intention (%)	TPP	16.16	21.68	38.04	31.37	25.56	23.28	33.91	35.00	32.05	32.37
	ITPP1	14.63	13.27	33.15	24.40	41.67	20.69	32.87	16.79	23.08	19.23
	ITPP2	17.07	17.80	-	-	-	-	22.15	15.71	-	-
	CONV	25.30	15.86	5.98	15.28	4.44	6.03	3.81	6.43	-	-
	PREM	17.68	21.68	11.14	17.43	12.22	33.33	2.42	21.07	10.26	13.78
	NONE	9.15	9.71	11.68	11.53	16.11	16.67	4.84	5.00	34.62	34.62
WTP (€/product)	TPP	3.71	3.38	2.75	2.21	4.48	3.70	15.40	15.68	14.86	14.75
	ITPP1	3.50	2.79	2.38	2.06	4.74	3.25	14.63	12.01	17.16	11.86
	ITPP2	3.71	1.86	-0.73	0.91	0.78	1.65	14.79	-0.72		
	CONV	2.87	2.01	1.41	1.33	2.68	3.71	2.11	6.85		

	PREM	**3.54**	**2.89**	**2.75**	**2.21**	**4.48**	**3.70**	**8.64**	**6.60**	**5.00**	**5.26**
High FN size		**55 consumers**		**47 consumers**		**30 consumers**		**52 consumers**		**18 consumers**	
Purchase intention (%)	**TPP**	**12.27**	**20.42**	**21.43[b]**	**27.23**	**21.67**	**14.58**	**31.01**	**27.65**	**32.64**	**35.42**
	ITPP1	**7.27[b]**	**11.97**	**34.82**	**22.32[b]**	**45.83**	**13.75**	**25.72**	**23.46**	**9.72**	**4.87[b]**
	ITPP2	**14.32[b]**	**16.76[b]**	**-**	**-**	**-**	**-**	**23.32**	**22.96**	**-**	**-**
	CONV	**26.59**	**20.42**	**10.27**	**11.60**	**2.08**	**10.83**	**11.78**	**10.37**	**-**	**-**
	PREM	**22.05**	**14.93**	**17.41**	**17.85**	**7.50**	**31.25**	**3.61**	**10.86**	**19.44**	**26.38**
	NONE	**17.50[a]**	**14.93[a]**	**16.07**	**20.98[a]**	**22.92**	**29.58[a]**	**4.57**	**4.69**	**38.19**	**33.33[a]**
WTP (€/product)	**TPP**	**2.88 [b]**	**3.43**	**2.37 [b]**	**2.06**	**4.13**	**3.14**	**15.33**	**14.31**	**14.6**	**14.66**
	ITPP1	**2.88 [b]**	**2.41**	**2.37**	**1.90 [b]**	**4.79**	**2.83**	**13.84**	**12.70**	**13.98**	**4.90 [b]**
	ITPP2	**3.34 [b]**	**1.89**	**-0.42**	**0.69**	**0.60**	**1.62**	**14.51**	**0.64**		
	CONV	**2.86**	**2.88**	**1.51**	**1.26**	**2.40**	**3.46**	**2.60**	**2.90**		
	PREM	**3.68**	**2.19**	**2.37**	**2.06**	**4.13**	**3.14**	**10.29**	**7.09**	**6.59**	**7.08**

[a, b]: Refer to significant difference between the Low and high FN clusters (column comparison) for the analysed products
Shadowed cells highlight the significant difference between clusters

The FN trait was also related to the non-hypothetical WTP (Table 8). Results showed that in Croatia and Slovenia the low and high FN clusters had similar WTP for the TPP and the ITPP before and after the hedonic evaluation. In Italy and France, results showed only significant relation between the FN and the WTP for the ITPP after the hedonic evaluation. In Spain, the relation between the FN and WTP was highly significant before the hedonic evaluation, while it turned to be non-significant after the hedonic evaluation test. The WTP association with the FN was able to extract more significant relations. This result showed the relevance of the price attribute when analysing the relation of FN and food choice.

3.2.2 FN and the expected liking

The FN was associated with two different ways of eliciting consumers expected liking; the traditional 9-points hedonic scale and the direct numerical probability alternative. Focusing on the pork products and innovations from the untapped breeds, results (Table 9) showed a clear pattern in all countries; consumers with low FN exhibited higher expected liking probabilities and expected liking scores for the TPP and ITPP. However, despite of this apparent trend, non-significant differences were found in Croatia, Slovenia and France for the TPP, and in Slovenia and Croatia for the ITPP.
However, significant differences were found in Spain for the TPP and the ITPP and in Italy for the ITPP. The direct numerical probability elicitation alternative was able to extract more variability than the traditional 9-points hedonic scale.

4. Discussion

4.1 Reliability of the adapted FN scale across countries

After dropping the items 8 and 9, all PCAs were highly significant and the percentage of the total variance explained was also acceptable compared to other FN studies (Olabi *et al*., 2009 and Fernández-Ruiz *et al*., 2013). The variance by the first and second dimension in each country was also within the acceptable range of the FNS studies (Ritchey *et al*. 2003, Olabi *et al*., 2009 and Fernández-Ruiz *et al*., 2013). The adapted FN scale has been showed to be a valid and reliable tool to extract consumers' FN in our analysed samples.

Table 9: The FN associations with the expected liking

Healthy and sensory perception	Spain			Italy			Slovenia			Croatia			France		
	Lw.	Av.	Hg.	Lw.	Av.	Hg.	Lw.	Av.	Hg.	Lw.	Av.	Hg.	Lw.	Av.	Hg.
Probability liking expectation (TPP)	79.2[a]	70.4	64.4[b]	82.2[a]	74.1	69.3[b]	73.1[a]	73.3	67.6[a]	79.1[a]	80.5	74.1[a]	80.4[a]	74.8	70.5[a]
9-points liking expectations (TPP)	6.4[a]	6.7	5.9[a]	7.7[a]	7.4	7.1[b]	6.9[a]	6.8	7.1[a]	7.1[a]	7.3	6.7[a]	7.4[a]	6.9	6.8[a]
Probability liking expectation (ITPP1)	74.5[a]	66.0	59.7[b]	80.8[a]	71.7	69.6[b]	81.6[a]	80.5	74.1[a]	76.4[a]	75.2	66.5[a]	86.4[a]	78.9	73.0[b]
9-points liking expectations (ITPP1)	5.7[a]	5.9	5.6[a]	7.7[a]	7.3	7.4[a]	7.5[a]	7.2	7.3[a]	6.9[a]	6.6	6.1[a]	8.0[a]	7.5	7.3[b]
Probability liking expectation (ITPP2)	80.5[a]	65.5	63.4[b]							76.0[a]	72.3	63.4[b]			
9-points liking expectations (ITPP2)	6.3[a]	6.1	5.9[a]							7.2[a]	6.5	6.4[b]			
Probability liking expectation (CONV)	81.0[a]	69.7	70.8 [a]	51.4[a]	50.1[a]	53.4 [a]	45.1[a]	45.1	43.0[a]	35.5[a]	43.7	41.2[a]			
9-points liking expectations (CONV)	6.8 [a]	6.4[a]	6.6[a]	5.2[a]	5.3[a]	5.5[a]	4.4[a]	4.3	5.1[a]	4.9[a]	5.3	5.1[a]			
Probability liking expectation (PREM)	82.9[a]	79.1	71.2[a]	55.4[a]	57.4	55.5 [a]	57.6[a]	59.7	61.2 [a]	57.8[a]	57.5	57.4[a]	54.5[a]	48.2	51.4[a]
9-points liking expectations (PREM)	6.9[a]	6.4	6.8[a]	5.9[a]	6.0[a]	6.0[a]	5.9[a]	6.5	6.1[a]	6.3[a]	6.2	6.3[a]	5.2[a]	4.8	5.6[a]

[a, b] Denotes significant difference at 95% between clusters (shadowed cells) within case-study

Comparing the two method of eliciting consumers expected liking, results showed a perfect agreement in Slovenia, Croatia and France between both elicitation methods. Discarding items from the original FNS is not new because of the potential dissimilar interpretations of what consumers from different countries and culture may understand from the original statements (Ritchey *et al*., 2003). In their study, they dropped the items 5 and 9 due to a misinterpretation of statements (translation or other latent problems) in a Sweden case study. They also mentioned that the FNS is not unidimensional since the understanding of some items does not express the same phenomenon depending on the countries analysed. They even realized that a model based on 6 items only (1, 3, 4, 6, 7, and 10) may be applicable to understand consumer FN and reaction to novel food products when compared between countries (US, Sweden and Finland) as was applied in Alemu *et al*. (2016) who included only items that were deemed culture suitable for Kenyan consumers. Chen (2007) used only five items of the FNS (1, 2, 3, 7, 9) following Bredahl (2001). Van Wezemael *et al*. (2010) also used a reduced FNS form using 5 items obtaining good reliability of the scale. In all the cases, Damsbo-Svendsen *et al*. (2017) in their review stated that excluding items from the FNS may improve FN structure when used in several countries and recommended a prior evaluation of the content of items in the FNS and using only those that are relevant in each context.

4.2 The relation of FN with the non-hypothetical WTP, PI and expected liking

The FN was significantly associated to the expected PI when innovations consisted of enriching the products with unfamiliar components, but was irrelevant when innovations were simply a removal of relatively familiar ingredients. In all cases, there is non-significant tendency regarding the low FN consumers in revealing higher PI toward innovations. Only FN association was significant in Spain where innovations were relatively novel in patty products. Introducing only the breed type as differentiating attribute did not create major changes regarding the familiarity perception. The association of the FN with the expected WTP confirmed, in general, the previous results. However, including the price attribute in the food choice of the products with unfamiliar innovations in non-hypothetical frameworks could lead to unbiased association between FN and purchase expectation (Raciti 2016; Alemu *et al*., 2016; Schnettler *et al*., 2013).

It is also important to verify that some changes occurred regarding this association after the hedonic evaluation test. Several studies showed that the significance of the FN associations can vary according to experiment environment. The tasting experience with novel or unfamiliar flavours can produce changes in preference (Birch *et al*., 1987, Baba *et al*., 2016). Mustonen & Tuorila (2010) and Park & Cho (2016) showed that tasting education and the eating experience may reduce FN. This outcome appeared in the Spanish case study. After the hedonic evaluation, the association of the FN with the experienced PI and the experienced WTP for the ITPP1 (enriched with dietary fibber) turned to be non-significant. The same also occurred for the experienced WTP for the ITPP2 (enriched with natural antioxidant), highlighting in this case the relevance of considering also the WTP when consumers' purchase choice is related to FN. However, contrary to this outcome, the hedonic evaluation accentuated the difference of the PI and WTP between food neophobic and neophilic consumers in Italy and France. Theses changes are likely related to the fresh characteristic of the meat product (patty) used in Spain and to its lower value perception compared to the other processed products.

The findings from the estimated WTP may diverge from those obtained from the PI due the relevance of the price attribute in defining consumers' purchasing decision. The inclusion of a non-forced choice gave the consumers the opportunity to opt-out in their choice (Kallas, *et al.*, 2013). Thus, the WTP was able to capture higher preference variability between food neophobic and neophilic consumers. Considering the price information in drawing consumer's preferences is a key factor for understating the consumers' final decision towards food innovations.

Focusing on the hedonic evaluation, consumers with lowest FN showed the highest expected liking for the TPP and the ITPP in comparison to consumers with the highest FN, depending on the ITPP type and country as found in other several studies (Fernández-Ruiz *et al*. 2013; Laureati *et al*., 2018; Sanjuán-López *et al*., 2011). The relation of the liking expectation and the FN was emphasized when the direct numerical probability elicitation alternative was used, showing the capacity of this alternative to better capture FN heterogeneity. Innovations were more accepted by low FN consumers, in particular, when expectations were measured with the direct numerical probability method. Compared to the 9-points hedonic scale, the 100-points probabilistic outcomes were able to capture higher discrepancy and thus better confirming the FN relation with

the liking expectations. As commented by Preston and Colman (2000), longer scales may capture higher variability in relatively small samples.

5. Conclusions

Results showed a high non-hypothetical expected WTP and expected PI for products obtained from the unfamiliar pig breeds, revealing high potential for their market penetration. However, after the hedonic evaluation, the expected WTP for the proposed innovations were negatively disconfirmed in the majority of the cases in Spain, Italy, France, Slovenia and Croatia. Including the tasting experience in researches that focused on consumers WTP towards food innovation is highly important.

The difference between the expected and experienced WTP towards the innovations showed higher variability in Spain, Italy and France when compared to the difference between the expected and experienced PI. Our results highlight the relevance of the additional information gathered when the price attribute is considered in a non-hypothetical framework to define consumers' preference for food innovations.

Analysing the consumers' FN personality trait, consumers in France were the least food neophobic. The Spanish consumers exhibited higher FN than the Slovenian ones. Consumers in Italy and Croatia showed average FN scores. Focusing on the innovations, the FN was significantly more associated to the expected WTP than the expected PI variable. Consumers with low FN trait exhibited higher expected WTP, in particular for the unfamiliar innovations in Spain (enriched with natural source of dietary fibber and enriched with natural antioxidant) in the patty product. However, after the hedonic evaluation, the FN association appeared to be only significant with the experienced WTP for the innovations in Italy (without conserving agent) and in France (ripening time). Consumers with high FN tended to avoid purchasing any of the products proposed in the experiments by selecting more the option "neither of them". This relation was also accentuated after the hedonic evaluation in Italy and Slovenia.

An apparent trend appeared for the consumers with low FN who exhibited higher expected liking probabilities towards the products from the untapped pig breeds. In particular, it was significant for the innovations in Spain (dietary fibber and natural antioxidant), Italy (natural preserving agent) and France (ripening time). The direct

numerical probability elicitation was able to extract more variability than the traditional 9-points hedonic scale in associating the expected liking with FN.

Our study showed that the FN trait is likely to play, in some cases, a relevant role in defining the consumers' liking expectations, the non-hypothetical PI and WTP for the proposed food innovations. However, results clearly depended on the innovation and product types. It would be worth considering a classification of the food innovations regarding their familiarity and novelty, if they consist of a reduction or an enrichment and if are introduced in fresh or processed products when associated to FN. Future research with large samples, including hypothetical and non-hypothetical choice design are required to better identify more significant results.

Acknowledgements

This study has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 634476 (project acronym TREASURE). The content of this paper reflects only the author's view and the European Union Agency is not responsible for any use that may be made of the information it contains. Core financing of Slovenian Research Agency (grant P4-0133) for MČP and UT is acknowledged.

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Annex 2

Measuring Consumers' Preferences for Traditional and Innovative Pork Products

Published in *Agriculturae Conspectus Scientificus as*:
Kallas, Z. *et al*. (2017) 'Measuring consumers' preferences for traditional and innovative pork products', *Agriculturae Conspectus Scientificus*, 82(2 Special Issue 1).

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Personal contribution: Italian products manufacturing and collaboration in the execution of the consumer test and results discussion.

ORIGINAL SCIENTIFIC PAPER

Measuring Consumers' Preferences for Traditional and Innovative Pork Products

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Summary

In this research we proposed an integrated methodological approach to measure the "real" consumers' preference towards new Traditional (TPP) and Innovative Pork Products (ITPP) from three untapped pig breeds in Spain (*Porc Negre*), Italy (*Cinta Senese*) and Slovenia (*Krškopolje*). We first analyse consumers' perception towards the traditional concept in pork products. Results showed high preference heterogeneity amongst countries. After the eating experience, the expected preferences were affected significantly in particular in Italy and Slovenia. The likelihood to purchase the innovative pork products increased as well.

Key words

consumers' preference, consumers' acceptance, pork products

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Received: April 30, 2017 | Accepted: September 25, 2017

ACKNOWLEDGEMENTS
This study has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 634476 (project acronym TREASURE). The content of this paper reflects only the author's view and the European Union Agency is not responsible for any use that may be made of the information it contains.

Introduction

The analysis of consumers' preference towards traditional food products (TFP) and innovative food products (ITFP) is gaining relevance in the last decades. The European Parliament and the Council of the European Union (2012) identifies the TFP as a product with a proven usage on the domestic market for a period that allows transmission between generations; this period is to be at least 30years". Thus, analyzing consumers' perception and the consumer-driven definition towards the TFP is highly relevant to understand consumers' reaction to new products at market place (Balogh et al., 2016). The meat industry has started introducing innovations to improve the nutritional and health properties of meat processed products (Toldra & Reig, 2011). The demand for food products enriched with natural ingredients that provide health benefits is increasing. Thus, the production of healthier products is one of the innovations that is being continuously incorporated into processed meat products. The innovative pork products with healthy benefit are still at an early stage, with only a few products launched on the global market (Grasso et al., 2014).

In this context, consumers' preferences and purchase intention towards pork products obtained from three untapped pig breeds in Spain (Porc Negre), Italy (Cinta Senese) and Slovenia (Krskopolje) were analysed. In each case study, and according to each market interest and potential expected demand, specific products and innovations were identified. Table 1 shows a summary of the selected products.

Within the range of techniques that analyze preferences, several alternatives are available. The Choice Experiment (CE) is one of the most used in the exploration of individuals' preferences (Alfens, 2004). This method has demonstrated its capacity to analyze preferences for "complex goods" such as food products. The choice experiment aims at identifying the individual's indirect utility function associated with attributes of products by examining the trade-offs consumers make when making choices at the retail outlet. Thus, several alternatives (products) that are described by several attributes (breed, innovations and price) with varying levels (breed types, innovation types, price levels) are presented to the respondents in an array of choice sets or cards. These cards show different competing products at different prices. Within each choice card, respondents are then asked to select his/her preferred product (alternative) or to rank the products from the best to worst product, thereby revealing his/ her preference for certain

attributes and levels. Subsequently, the willingness to pay for the different attributes (and consequently the breed or the innovation) can be indirectly recovered from respondents' choices. The conceptual foundations of CE rely on Lancaster's Theory of Value (Lancaster, 1966), which proposes that utilities for goods can be decomposed into separable utilities for their characteristics or attributes, and Random Utility Theory (Thurstone, 1927), which explains the dominance judgments made between pairs of offerings.

Consumers' preferences were analyzed before (expected preference) and after the eating experience (experienced preferences). We applied a real Discrete Choice Experiments (DCE) in order to avoid the hypothetical bias. In a hypothetical DCE, consumers choose their preferred product from each choice set without any real consequence derived from this selection (i.e. they choose one product, but they do not have to buy it). Several studies have criticized this approach since some results seem to show divergence between what consumers select as their preferred product and what they would purchase in real life, posing under question the validity of hypothetical experiments (Loomis, 2014). Previous studies indicate that individuals, in general, respond to hypothetical scenarios surveys differently from the way they act in real life (Murphy et al., 2005). It is quite common to find that individuals say they are willing to pay higher prices than those that they are really willing to pay. This is due to the difficulty in calculating the exact impact of these higher expenses on the household economy. It is easy to be generous when in reality one does not need to pay more.

To overcome such disparity, some alternatives have been developed in the literature. One of the most convincing ones is the inclusion of economic incentives by creating a real shopping scenario. In such a situation consumers have an incentive to behave truthfully and to choose the products he/she would actually buy in a real setting. To create a real shopping scenario, consumers are usually unexpectedly informed that they will be rewarded with some additional income. Participants who participate in the survey should "purchase" their preferred product and pay its price.

Material and methods

We first analyzed the consumer-driven definition of the traditional concept in pork products on the basis of literature. Twenty-two statements were evaluated using 9-point

Likert scale ("1" disagree very strongly, "2" disagree strongly, "3" disagree moderately, "4" disagree slightly, "5" neutral, "6" agree slightly, "7" agree moderately, "8" agree strongly and "9" agree very strongly). The most relevant statements were: anchored in the past (Guerrero et al., 2009), tied to specific localities, regions or countries and typically evoke strong memories of childhood (Cerjak et al., 2014; Rudawska, 2014), passed from one generation to generation and usually in a domestic setting or by artisans (Guerrero et al., 2009), possess distinctive and positive sensory merits (Molnar et al., 2011, Almli et al., 2011), genuine and au-thentic (Tregear et al., 1998, Guerrero et al., 2009), part of an area's gastronomic heritage (Guerrero et al., 2009), familiarity and the natural content (Pieniak et al., 2009).

The relevance of the methodological approach proposed in this study is its ability to mimic how consumers react in "real life" when facing a novel/new product. When consumers face new/novel products in a retail outlet, they create expectations on the basis of their past experience and available information. The analysis of consumers' preferences following this experimental design (i.e. before tasting the product) is identified as "expected preferences" and are elicited taking into account consumers' choices. The range of products offered to consumers includes the existing ones in the market and the new ones in order to simulate a real purchasing situation. In a subsequent step, consumers taste the new/novel products and existing products and then they create a subjective "sensory experience" (consumer acceptance analysis). This eating experience in real life is crucial as it allows consumers to decide if they are willing to repurchase the product. After tasting the products, the decision to repurchase the product might be affected. Thus, the preferences analysis is repeated because the sensory experience may result in agreement or disagreement with what they expected and therefore the decision to repurchase the product will be different among them. The analysis of preferences at this point is identified as "experienced preferences". Therefore, we followed a combination of the preferences analysis (real willingness to purchase) and the consumer acceptance (sensory liking). The complementarity of these analyses allowed us to analyze if the proposed products may reach successfully the market by comparing what consumers expect and what they experience after tasting the product.

For the choice sets construction, we put the different TPPs and ITPPs jointly with two conventional pork products that are actually sold in the markets but with two different

qualities: a regular one with low market price (CONV) and a premium one with the high market price (PREM). Each choice set contains the TPP, the ITPP, the CONV and the PREM products categories that appear at different price combinations. A NONE option was also offered if consumers reject to purchase any products. Data collection for consumers' acceptance and preference (real choice experiment), was carried out for the same 120 consumers in each case study. A quota sampling approach was used stratified by gender and age. Consumers eligible to participate were over 18 years of age who regularly purchase food and beverages and having purchased and consumed the products proposed in the last month. Consumers were economically "compensated" for their participation by direct payment of money delivered at the end of the experiment. At the end of the whole experiment, the amount of the "unexpected payment" was paid to consumers to participate in the real purchasing scenarios of the "products".

Results and discussions

As can be seen in Figure 1, we first identified the consumer-driven definition of the Traditional Pork Products concept. In general terms, the perception towards the Traditional Pork products were positive, showing high agreement level with the positive statements in all countries and low mean value for the negative statements. The traditional concepts in all countries was highly identified as part of area gastronomic heritage in agreement with the results found in Guerrero et al., (2009). It was also recognised the role of this type of products in maintaining the local economies and the highest perceived quality they have. There was also an agreement on their handmade and artisanal production system. However, observed heterogeneity is found, especially regarding the safety aspect. While these types of products are considered safe in Slovenia and Spain, they received lower score in Italy. The same for the healthy perception, where in Slovenia were considered less healthy than in Italy and Spain. Further analysis is needed to understand this observed heterogeneity with regards to the socioeconomic and cultural variables.

The preliminary results regarding the expected and the experienced purchase intention extracted are presented in Figure 2. It is worth mentioning that these results were extracted from only one card from the DCE where the products appeared to have the same price.

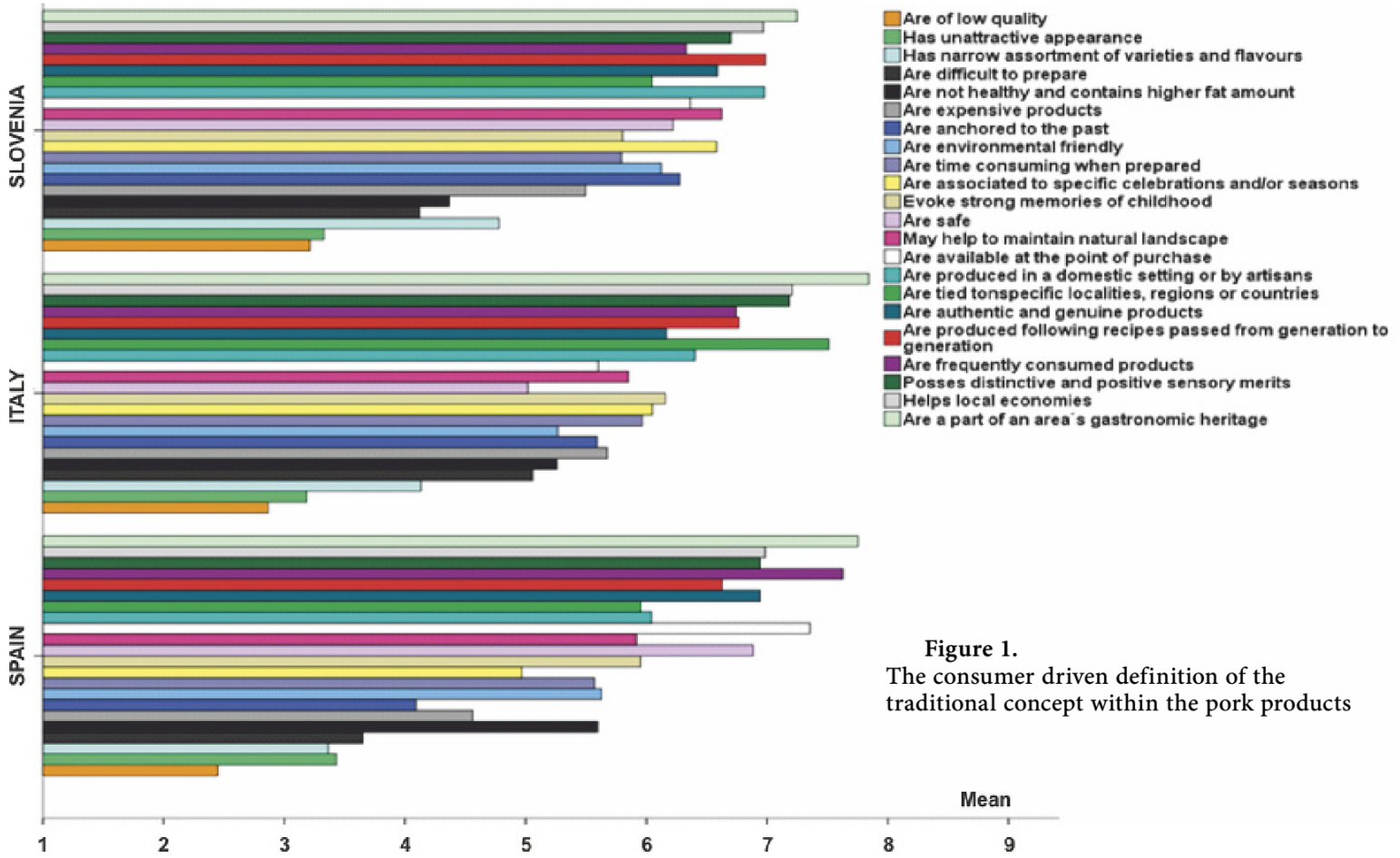

Figure 1.
The consumer driven definition of the traditional concept within the pork products

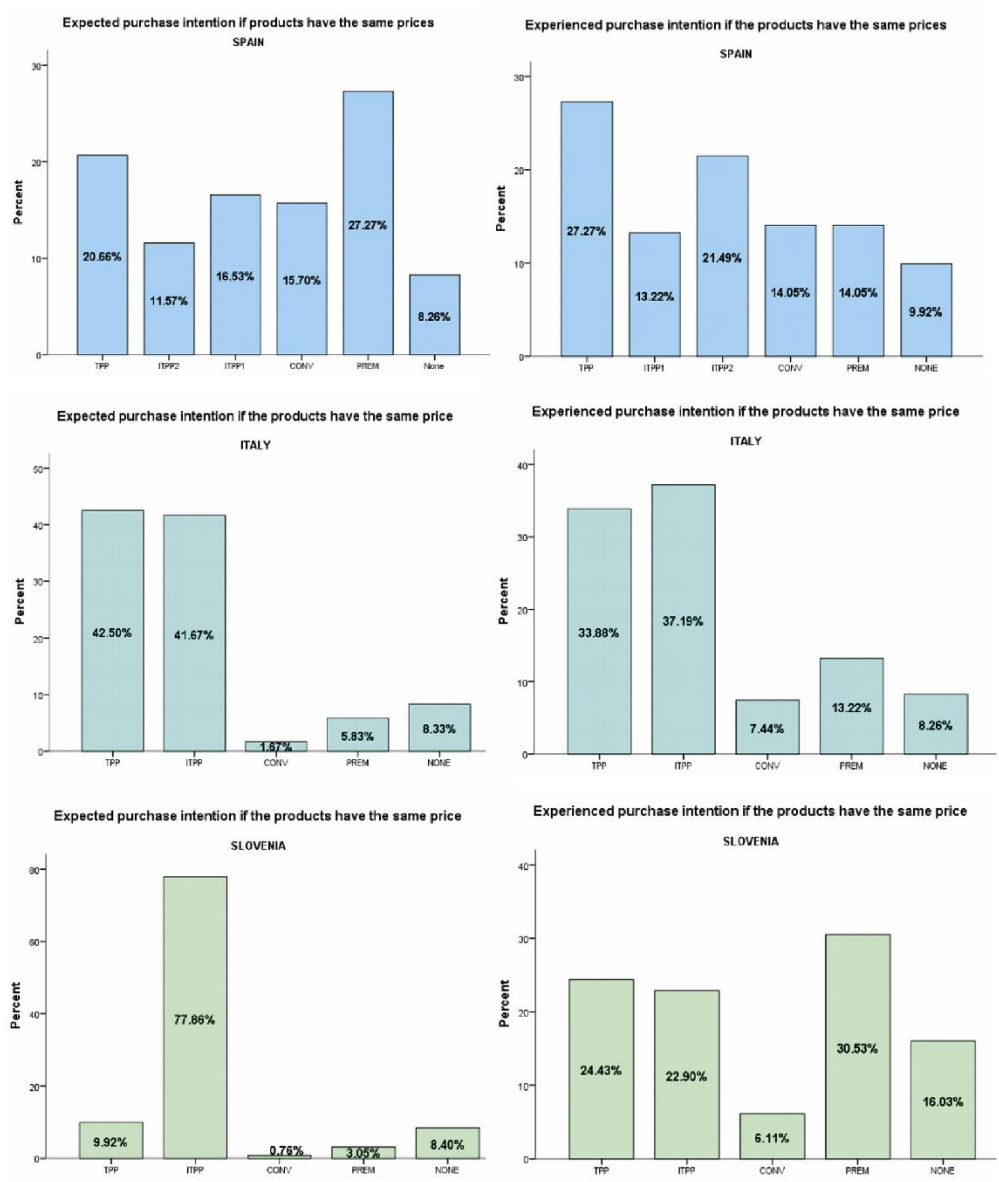

Figure 2. Expected and experienced purchase intention if the products have the same price

As can be seen in Figure 2, two preliminary results can be retrieved. First, the percentage of respondents who showed the intention to purchase the TPPs and the ITPPs after the eating ex-perience increased in Spain but decreased in Italy and Slovenia. Results from the sensory experiment may shed light on these finding. However, these results are beyond the objective of this paper. Second, the total percentage of purchase intention of

the TPP and the ITPP in Italy and Slovenia was relatively high in comparison to the other products offered in the choice sets and also in comparison to the Spanish TPP and ITPP products. It seems that innovations regarding the expected purchase intention in Slovenia and Italy were more accepted in comparison to the Spanish case study. However, innovations regarding the experienced purchase intention were more accepted in Italy compared to Spain and Slovenia.

In all cases, results should be treated carefully because they represent only a part of the choice experiment results, and because of the relatively small samples in each case study. Furthermore, results should be interpreted as specific to each case study due to the difference in products, the price and innovations levels. Future research with large sample should be carried out to better shed light on conclusion with more significant results.

Conclusions

We analysed the "real" purchase intention towards traditional and innovative pork products before and after the eating experience. The preliminary results of the expected real purchase intention showed that the TPP and the ITPP are likely to be purchased if the decision is only based on the products' characteristics. However, the expected purchase intention decreased in Slovenia and Italy while increased in Spain after the eating experience. Further analysis is need from the sensory point of view to shed light on these results.

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CONCLUSIONS AND FUTURE PERSPECTIVES

Cinta Senese pork chain is a long-standing reality of the Tuscan territory, its stakeholder can rely on a rooted know-how both at rearing and manufacturing levels. However, the changing market and the new challenges for environmental sustainability, animal welfare and human health, are requiring a new approach to swine production, even for a traditional system as the Cinta Senese one. The few studies on local pig breeds and on Cinta Senese in this specific case, offer several possibilities of intervention in the production system to enhance its economic and environmental sustainability, but also to preserve its traditional features. Decades of research on improved pig breeds have identified the best energy:protein ratio for each stage of growth, as well as the optimal intake of essential AA, to optimize the diet utilization and to enhance lean genotypes' performances. The results obtained from Trial 1 have shown that, for Cinta Senese growing pigs, a better feeding management can be applied by lowering the dietary CP up to 12%. The identified protein level is far lower than the CP content currently used by farmers, that is based on lean-genotypes formulations. Supplying the adequate dietary protein to fulfil animal's requirements had positive effects on the overall performances and on N excretions, that resulted markedly lower for the animals fed the lower CP formulations. This can have positive impacts on animal welfare and environment, but also on farmer's income. These results, together with those obtained by the earlier studies on Cinta Senese growing-fattening stages, could constitute an opportunity for farmers to start applying the knowledge acquired for selected genotypes also on local breeds and to move towards novel feeding strategies as the precision feeding. In this respect, further researches are needed to better identify the protein requirements for each single stage of growth; similarly, the best protein:energy ratio and Lysine:energy ratio might be interesting parameters to be evaluated.

Improving the feeding management can help Cinta Senese pork chain to address the environmental issue, but the changing demand for final products cannot be ignored. Consumers' concerns for food safety is arising, as well as the mistrust for pork products. For instance, the Cinta Senese fresh meat market is struggling because fresh meat is perceived too fat by most of the consumers. Inversely, processed products are well-

known and appreciated for their high-quality and sensorial characteristics, but the presence of nitrite and nitrate in them, started rising concerns on their safety. Trial 2 has tried to respond to these different matters by proposing novel and healthier ingredients in a fresh product (the burger) and in a cured product (dry-fermented sausage). In the former experiment, the innovation was aimed to enhance the lipid fraction by adding long-chained omega-3 PUFA. The diffusion of burger in the last years, made this product an interesting opportunity to raise awareness of Cinta Senese in a broader market, while the microencapsulation technique resulted effective in increasing the product's omega-3 content also after storage and cooking, protecting the added PUFA from lipid oxidation. Moreover, the use of deodorized fish oil and of microencapsulation have preserved the sensory traits of the products. The novelty proposed for the cured product was to replace the sodium nitrite by two different mixture of natural antioxidants, consisting of: i) grape seed extract and olive pomace hydroxytyrosol (GSE); ii) chestnut extract and olive pomace hydroxytyrosol (CHE). The results on volatile compounds' profile suggested a greater antimicrobial activity of natural antioxidant mixtures compared to sodium nitrite, nevertheless none of the main foodborne pathogens were found in any sample. No significant differences among treatments were found for lipid oxidation, even if lipid auto-oxidation volatile compounds suggested a slightly lower antioxidant activity of the natural antioxidants compared to sodium nitrite. Despite the differences in single volatile compounds' abundances, the replacement did not affect the overall aroma profile, as outlined by GC-O results and sensory analysis. The effects of the natural antioxidants on dry-fermented sausages' microbiota should be studied in depth, but the results so far, indicated that the tested antioxidants are valid alternatives to sodium nitrite. In the consumers studies, where the above-mentioned cured product was involved, the expected purchase intention showed a relatively high preference for the local pig breed's product, with or without novel ingredients, with respect to conventional and top-quality products of commercial breeds. After the hedonic evaluation, the willingness to pay for the traditional product decreased contrarily to the natural antioxidant product's, that remained unchanged. These results confirm the rising interest for local breeds and they suggest that Cinta Senese pork chain has an untapped potential at market level, that should be exploit by optimizing its management at farm level and through the development of more appealing products.

ACKNOWLEDGMENTS

Firstly, I would like to thank my tutor Prof. Carolina Pugliese for the support given during these three years, for her patience and motivation and for her priceless help in all the stages of my PhD.

Besides my tutor, I would like to thank my thesis committee: Prof. Giuliana Parisi and Prof. Arianna Buccioni for the time spent in improving my work, for their valuable advices that incented me to widen my research from various perspectives.

My sincere thanks also go to Prof. Oreste Franci for the help in interpreting the data, to Dr. Francesco Sirtori, without whose precious support it would not be possible to complete this research and to the whole technical staff, Doria Benevenuti, Ilaria Galigani, Antonio Pezzati, Antonio Bonelli and Silvano Lancini, for the support in the laboratory work and field trial.

I would also to thank Prof. Teresa Antequera and Universidad de Extremadura for the time spent in Càceres, without the enjoyable and interesting experience in her laboratory I probably would not have chosen this path, and Prof. Monica Flores and IATA-CSIC, for the valuable lessons and for her support along all my stay in Valencia.

I thank my PhD colleagues for having shared the difficulties of this journey, making it lighter. A special thanks goes also to my foreign friends who have been my family away from home and have made my stay abroad unforgettable.

I would like to thank European Union's Horizon 2020 research and innovation program for having funded the research (grant agreement No 634476, acronym TREASURE).

Last but not the least, I would like to thank my family and friends for their support in tough times and for putting up with me throughout this PhD….and before.