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Doctoral Thesis

INNOVATION TECHNOLOGY IN FEED FORMULATION AND PRODUCTION

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Abstract

The general aim of this thesis was to investigate new technology in feed formulation and production. For this purpose several aspects in the field of feed production were investigated. Specifically, during the course of this PhD program, three main sub aims have been addressed: i) evaluation of new ingredients, i.e., insect materials, in feed formulation and the impact of feed technology on their nutritional value; ii) improvements in the official methods for detecting Processed Animal Proteins (PAPs), and iii) investigation of the innovation, research, and development needs in in the feed industry in two selected areas.

With respect to the first sub aim, fresh insect (Hermetia illucens) material was evaluated as an alternative protein source in experimental feed. Differents experimental formulae containing insect material were tested under differing extrusion conditions. Extruded feed was also evaluated for digestibility. The results indicate that fresh Hermetia illucens material can be efficiently included in experimental extruded feed containing 25% insect material and 75% wheat. Technological treatment, i.e., extrusion, increased in vitro organic matter digestibility, and did not affect protein digestibility.

With regard to the second sub-aim the basic assumption was that insect material, if authorized, should be considered as animal material. Accordingly, the second sub aim of the thesis focused on implementing existing methods for processing animal protein and investigating their potential in tracing and characterizing insect material. In this scenario, experiments were aimed toward improving the official microscopy techniques for detecting processed animal proteins (PAP) by combining those with image analysis (IA) technology. The studies conducted aim to i) characterize fish meal material in compound feed (i.e., aquafeed), ii) identify specific selected markers able to efficiently distinguish between fish and terrestrial materials, iii) distinguish between mammalian materials, and iv) verify the applicability of the method for identifying insect material in feed. The results obtained in this context indicated that even though microscopy seems to be a promising approach for identifying both animal proteins and insect material, using microscopy alone has some limitations; therefore, a combined approach with other methods (i.e., PCR) is recommended.

With regard to the third sub aim, research and development needs and innovation in the feed industry, the results of a targeted survey conducted in two countries (Italy and Serbia) showed that innovation in raw materials is a key factor for large multinational industries. In contrast, the survey results obtained from small and medium feed companies are quite different; for these companies, cost reduction, decreased energy consumption, improved quality, improved market image, development of new markets and satisfying market demand are much more important.

By combining the results obtained from the various studies described in this paper, it can be concluded that: i) Insects show great potential as a protein source in animal feeds. Specific selected feed technologies, such as extrusion, can be useful in making such feeds convenient and safe to use. ii) Assuming that insect material will be authorized for use in animal feeds, existing methods for processing animal proteins may represent an advantageous starting point. Further investigation and implementation of methods of analysis is still required. iii) Even though insect materials as animal nutrition can be considered as a "hot topic" from a scientific point of view, not everyone in the feed sector seems to be aware of the issue. Addressing "new ingredients", co- and by- products remain the main categories in the feed sector mind consciousness.

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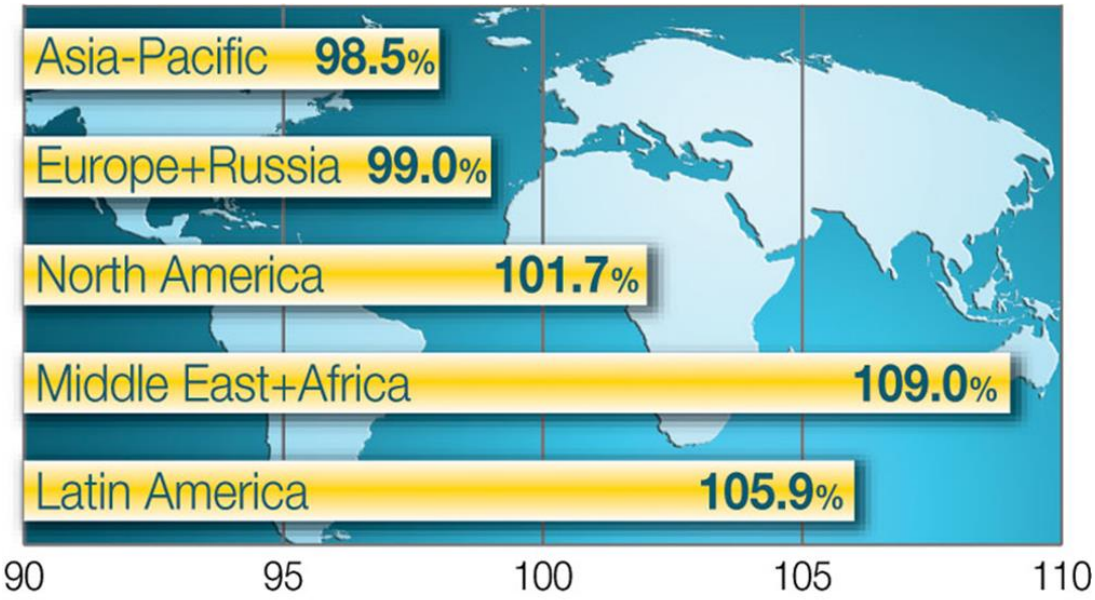
Introduction

An overview of the feed sector

Recent feed sector updates (FEFAC, 2015) report that the EU-28 contributes 16% of global feed production, estimated at approximately 964 mio. tons. Considering the global scenario, in 2014 Latin America, and Middle East and Africa have shown the highest increase in compound feed production –versus to 2013 volumes- compared to the other world regions. By contrast a slightly decrease for the same figures has been observed for Asia-Pacific and Europe/Russia regions (figure 1- Best, 2015). Nevertheless, the EU feed sector is the most important agricultural input industry in Europe and is an essential supply partner to the livestock industry (EUFETEC, 2013).

FIGURE 1 2013-2014 VARIATIONS IN COMPOUND FEED PRODUCTION IN DIFFERENT WORLD REGIONS (FROM BEST, 2015)



As reported in the latest review of livestock production and trade, more than 170 million tons of meat and other animal products were produced in the EU-28. To sustain this scale of livestock production, the EU-28 consumed 475 million tons of feed a year, of which half consists of roughage, 10% is farm-produced grains, 10% is purchased feed materials and the remaining 30% is industrial compound feed (FEFAC, 2015) (see details in figures 2 and 3). Compound feed production in the EU-28 decreased slightly, by 0.5%, in 2014 to 153.4 mio. t. Pig feed production fell by 1.2% for the third consecutive year, whereas poultry and layer feed increased slightly (+0.3%), confirming their positions in the leading compound feed segment, slightly above pig feed. Cattle feed production also decreased by 1.2% (FEFAC, 2015). Germany and Italy recorded an increase in feed production (+ 2.3% and + 0.3%, respectively), while in the UK, France, the

Netherlands and Spain, production decreased by 1.6%, 0.7%, 4.5% and 0.8%, respectively (AgroNotizie, 2015).

FIGURE 2 EU-28 LIVESTOCK SOURCING IN FEEDINGSTUFFS - 475 MIO. T IN 2014

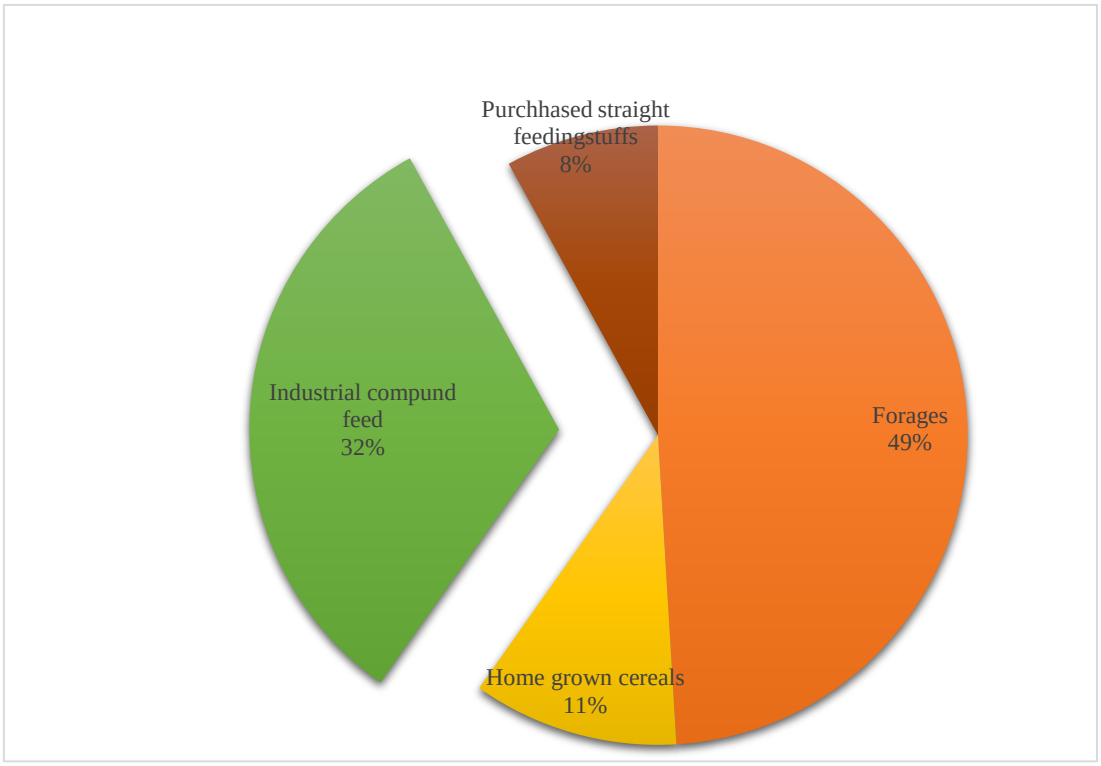
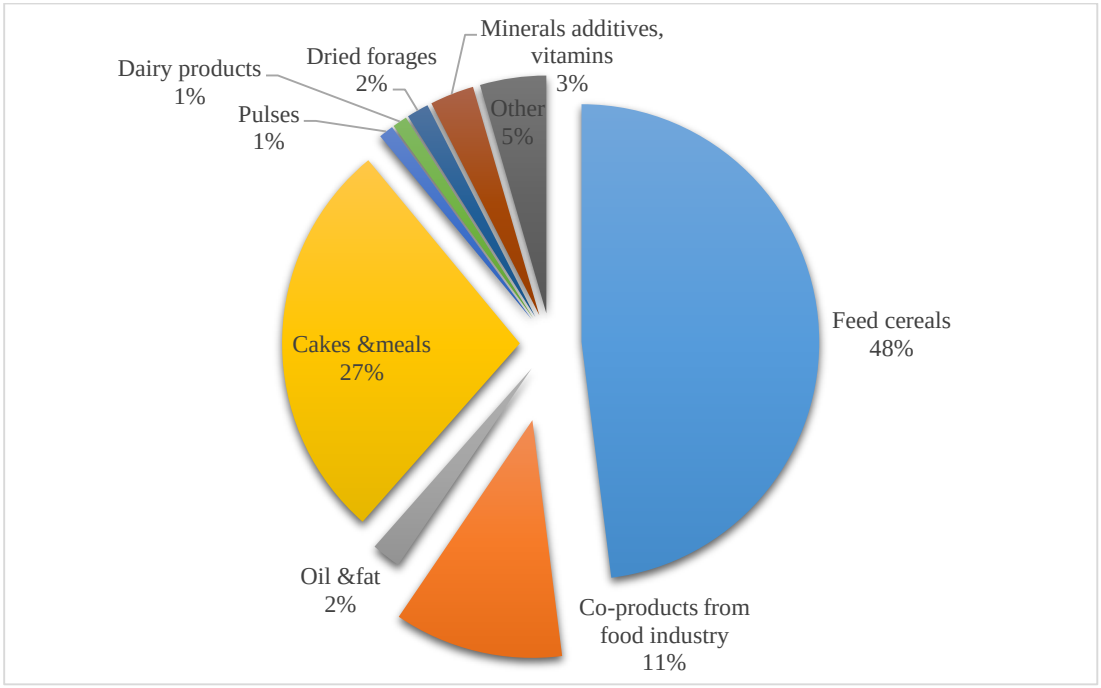


FIGURE 3 INDUSTRIAL COMPOUND FEED PRODUCTION IN THE EU-28 IN 2014 153.4 MIO. T (PER CATEGORY)



Notably, by themselves, the production of these six countries represents over 70% of total feed production among the EU-28 (AgroNotizie, 2015). To summarize the EU scenario, most EU member countries' production changed within a range of -3/+3%, with the noticeable exceptions of Ireland (-11%), due to lower demand for cattle feed, and Poland (+7%), a figure boosted by the demand for poultry feed. Germany's position as the leading EU country in terms of total compound feed strengthened; it is ahead of France and Spain, which are shoulder to shoulder in second place. Germany is the leading cattle and pig feed producer, while France maintains the lead in poultry feed production.

Despite the huge variations in feed material prices in recent years, the proportion of feed materials per category has remained relatively stable (48% for cereals, 27.5% for oilseed meal). However, this does not reflect the significant changes for some feed materials, e.g., corn gluten feed or dried distiller grains—usually imported from the USA—, which have almost disappeared since 2007 due to repeated trade disruptions caused by asynchronous authorizations of GM crops. Since the Mac Sharry reform in 1991, the average inclusion rate of cereals increased from 32 to 48%. On the other hand, tapioca, one of the most important substitutes for cereals in the 1980s, disappeared completely from the diets. Animal proteins, which in the past represented up to 2% of feed materials, were banned in 2001 and have been mostly replaced by soybean meal (FEFAC, 2015).

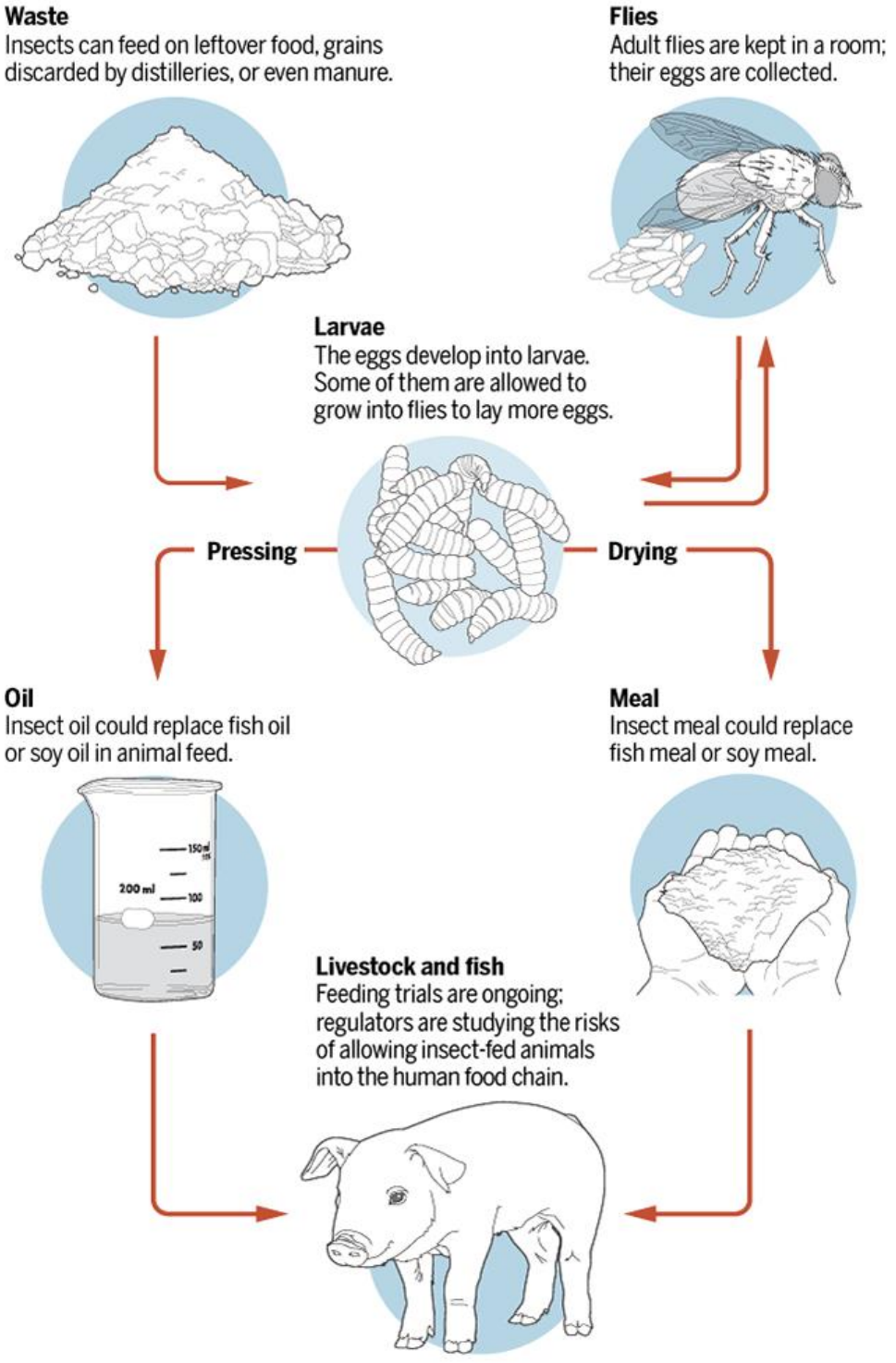
Livestock production: recent trends, future prospects

It is widely accepted that by 2050 the world will have a population of 9 billion people. To accommodate this number, current food production will need to nearly double. Because of increasing incomes, urbanization, environmental concerns, nutritional concerns and other anthropogenic pressures, the global food system is undergoing a profound change. There has been a major shift towards diets with increased consumption of animal products, and this change is likely to continue in the coming decades. The demand for meat and milk is expected to be 58% and 70% higher in 2050, respectively, than the levels were in 2010, and a large part of this increase will originate from developing countries (FAO, 2011). In this context, agriculture and animal production play a leading role in global food security.

The animal feed industry makes a crucial contribution to the global food industry. Feed is the largest and most important component for ensuring safe, abundant and affordable animal proteins. World compound feed production is fast approaching an estimated 1 billion tons annually. Global commercial feed manufacturing generates an estimated annual turnover of over US $370 billion (IFIF, 2014). According to the UN Food and Agricultural Organization (FAO, 2013) and IFIF

(2013) estimations, animal protein production will double for meats (poultry/swine/beef), as well as dairy, and fish production will almost triple by 2050. However, there are many concerns about the environmental sustainability of our current animal-based food production levels (Van Huis, 2013).

FIGURE 4 BIOCONVERSION TECHNOLOGY USING DETRITIVOROUS INSECTS PROPOSED FOR PRODUCING INNOVATIVE AND HIGHLY SUSTAINABLE PROTEIN AND OIL FOR AQUACULTURE AND LIVESTOCK FEEDS (MODIFIED FROM KUPFERSCHMIDT, 2015).



Protein sources traditionally used in feed production are related to sustainability issues, such as overexploitation and deforestation (Van Huis, 2013). Rapid growth of the aquaculture industry and the increasing demand for fish meal and fish oil (largely used in aquafeed) have resulted in rapid price increases (Jackson, 2012; Koeleman, 2015). At the same time, current protein sources such as soy are becoming more expensive. This explains why many companies are looking for protein alternatives to make animal feed more sustainable and affordable for farmers. Koeleman (2015) reported on a Malaysian company that developed a bioconversion technology to process organic side streams into insect-based products and bio-fertilizer. Using this technology, the company is able to make use of large-scale bioconversion techniques that use detritivorous insects, enabling production of innovative and highly sustainable protein and oil for aquaculture and livestock feeds (example in figure 4). Insect material in general—and high-protein insect meal in particular—represents an ideal alternative to fish meal, which is a key component of aquafeed and accounts for a huge import bill (Koeleman, 2015). Fish meal material is growing scarce because it is produced by only a few operators around the globe. Nevertheless, to meet the increasing demand for high-value protein meals, the EU Commission has re-authorized the use of non-ruminant meals in fish feed. The latter stems from a recent revision of the feed ban rules. Regulation (EU) No 56/2013 (European Commission, 2013a), which amends various earlier restrictions concerning the prevention, control and eradication of certain TSEs, was published in January 2013. The most significant amendment introduced by this rule is that from 1 June 2013, processed animal protein (PAP) from non-ruminants has been re-authorized for use as either feed or feed ingredients in aquaculture. The reintroduction of PAP from non-ruminants has also been made possible, thanks to the development of a consistent technique to identify and quantify levels of PAP in compound feeds. Currently, the EU uses two official control methods for detecting animal proteins in feed, namely, polymerase chain reaction (PCR), which delivers information on the species origin of the detected PAP, and light microscopy (European Commission, 2009a; European Commission, 2013c). Both methods were validated for proper implementation of the feed ban. Light microscopy in combination with computer image analysis (IA), which is based on the identification of bone particles or tissue in feedstuffs, has also been proposed (Pinotti et. al., 2013). These studies' findings indicated that the use of microscopy in association with computer image analysis for identifying PAP origins appears promising, especially when used as a complementary method for DNA-based methods. Therefore, the implementation of microscopy/image analysis techniques will be a key factor in view of a possible re-introduction of non-ruminant PAP in feed (IFFO, 2013). Such a reintroduction would also enable the EU to reduce its dependence on other sources of proteins.

Novel alternative protein sources

Based on the future feed and food scenarios worldwide, demand for food and feed is predicted to increase by as much as 70%, placing added pressure on already scarce agricultural resources (FAO, 2009; Pinotti et al., 2014). In particular, there will be a continued increase in global meat demand as developing countries undergo shifts in dietary habits that are associated with rapid urbanization and economic growth. The rearing of livestock for meat already places a considerable strain on global land and water use, and at present, much of the protein produced for livestock feed comes from unsustainable and environmentally damaging sources (IEEP, 2009). Ultimately, to meet the considerable challenges of assuring food security for the future, it is imperative that alternative, sustainable sources of protein be found, both for direct human consumption (figure 5) and for use in animal feed. Protein derived from insects represents one possible solution. The use of insects as food and feed has proved to be relevant, mainly due to the rising costs of major protein sources for animal feed (such as fish and soybean meal), food and feed insecurity, environmental pressures, population growth and the increasing demand for animal protein (meat, fish, dairy products, eggs, etc..) among the world's expanding middle classes (Makkar, 2014; Barroso, 2014; van Huis 2013; Veldkamp et al. 2012 Sánchez-Muros et al., 2014; Rumpold and Schlüter, 2013). In light of this, the concept of micro-livestock is emerging around the globe.

In several EU Member States, experimental insect rearing has already begun, intended for eventual use as a feed ingredient for farmed animals, and studies have shown that farmed insects could represent a sustainable alternative to conventional sources of animal proteins destined for feed (van Huis, 2013; Rumpold and Schlüter 2013; FAO 2013). Considering the present scenario the use of novel alternative and sustainable protein sources has been also proposed as a viable solution in the short term. In this respect, insects could provide an alternative animal protein source. Edible insects have always been a part of some human diets, but some societies have developed a degree of distaste for their consumption. Even in these societies, insects in animal feed can be an attractive feed option as a substitute for traditional protein sources.

FIGURE 5 RECORDED NUMBER OF EDIBLE SPECIES OF INSECT BY COUNTRY (FROM FAO, 2013)

A large number of studies were aimed at investigating the chemical composition and the nutritional value of several insect species (table 1). In general, these studies concluded that insects could serve as protein and energy supplements for animals. In fact, insects contain large amounts of protein, ranging from 20 to 70% on a dry matter basis (Sánchez-Muros et al., 2014; Rumpold & Schlüter, 2013; Odesanya et al., 2011). Moreover, because of their amino acid profiles, insects are considered a high-value protein source, comparable with fish meal and soy (Finke 2013, St-Hilaire, 2007). Furthermore, the presence of antinutritional factors in insects has not been reported in the literature.

However, insect exoskeletons and maggot cuticles both contain the polysaccharide chitin (Finke, 2007; Yi et al., 2013; Cauchie, 2001), which might affect the digestibility and utilization of other nutrients (Diener et al., 2009; Shiau and Yu, 1999). Nevertheless, chitinolytic activity has been observed in fish, where this nutrient might contribute to energy intake (Fines and Holt, 2010; Goodrich and Morita, 1977a,b). It has been reported that broiler chickens can also secrete chitinase in the gizzard (e.g., Han et al., 1997; 2000). In the case of pigs, no information is available concerning chitinase synthesis or secretion, but swine intestinal microbiota have been found to produce chitinolytic enzymes (Šimůnek et al., 2001). In this sense, the ingested chitin might contribute to nutritional value, and in turn, to energy intake in some farm animals.

TABLE 1 PROXIMATE ANALYSIS (% DRY MATTER) OF SELECTED INSECTS (INCLUDING SAGE OF DEVELOPMENT AND ORIGIN OF THE SPECIES ANALYZED), FISH MEAL AND SOYBEAN MEAL. VALUES ARE MEANS ± STANDARD DEVIATION. EE = CRUDE FAT. CP = PRUDE PROTEIN. NFE = NITROGEN-FREE EXTRACT (MODIFIED FORM BARROSO ET AL. 2014).

Species	Stage	Origin	Ash	EE	CP	NFE
Phyllognathus excavatus	Adult	Free-ranging	7.8 ± 0.2	15.9 ± 1.4	65.7 ± 1.3	10.6 ± 0.1
Rhynchophorus ferrugineus	Larvae	Free-ranging	6.6 ± 0.6	11.8 ± 1.5	34.6 ± 0.3	47 ± 1.3
Tenebrio mollitor	Larvae	Captivity	3.5 ± 0.2	30.1 ± 0.7	58.4 ± 0.4	8 ± 0.2
Zophoba morio	Larvae	Captivity	2.5 ±0.3	38 ± 0.3	53.5 ±0.4	6 ± 1.1
Calliphora vicina	Larvae	Captivity	8 ± 0.1	20.1 ± 0.7	48.3 ± 0.9	23.6 ± 0.1
Chrysomya megacephala	Larvae	Captivity	7.2 ± 0.1	27 ± 3.2	61.8 ± 0.3	4 ± 3.4
Chrysomya megacephala	Prepupae	Captivity	6.1 ± 0.1	16.5 ± 0	46.8 ± 1.1	30.6 ± 1.1
Eristalis tenax	Larvae	Captivity	13.9 ± 0.4	5.8 ± 0.6	40.9 ± 0.9	39.4 ± 1.1
Hermetia illucens	Larvae	Captivity	9.3 ± 0.3	18 ± 1.6	36.2 ± 0.3	36.5 ± 1
Hermetia illucens	Prepupae	Captivity	19.7 ± 0.1	15.6 ± 0.1	40.7 ± 0.4	24 ± 0.7
Lucilia sericata	Larvae	Captivity	4.9 ± 0.9	28.4 ± 1.5	53.5 ± 4.4	13.2 ± 4.6
Lucilia sericata	Prepupae	Captivity	4.9 ± 0.2	26.6 ± 1	59 ± 1.5	9.5 ± 0.1
Musca domestica	Larvae	Captivity	6.5 ± 1.5	31.3 ± 1.6	46.9 ± 4.1	15.3 ± 4
Musca domestica	Prepupae	Captivity	8.4 ± 2.9	33.7 ± 0.7	40.1 ± 0.4	17.8 ± 0.3
Protophormia terraenovae	Larvae	Captivity	3.9 ± 0.1	28.3 ± 0.6	46.3 ± 0.6	21.5 ± 0.1
Protophormia terraenovae	Prepupae	Captivity	8.8 ± 0.1	23.6 ± 0.3	56 ± 2	11.6 ± 2.2
Acheta domestica	Adult	Captivity	5.6 ± 0	15.9 ± 0.2	73.1 ± 3.3	5.4 ± 0.3
Anacridium aegyptium	Adult	Free-ranging	3.7 ± 0.1	17.6 ±0.2	66 ± 5	12.7 ± 4.8
Gryllus assimilis	Adult	Captivity	4.8 ± 0.1	23.2 ± 0.6	64.9 ± 0.5	7 ± 0.3
Heteracris littoralis	Adult	Free-ranging	5.1 ± 0.1	8.8 ± 0	74.4 ± 1	11.7 ± 1
Locusta migratoria	Adult	Captivity	4 ± 0	29.9 ± 0.5	58.5 ± 0.5	7.6 ± 0.1
Fish meal	-	-	18 ± 0.2	8.2 ± 0	73 ± 0.8	0.8 ± 0.7
Soybean meal	-	-	7.8 ± 0	3 ± 0	50.4 ± 0.2	38.8 ± 0.3

Several studies indicate that traditional protein and fat sources commonly used in feed formulation can be replaced by insects without any adverse effect on productive performance or product quality (Teotia and Miller 1973; Newton et al., 1977; Anand et al. 2008; Sealey et al. 2011; Fanimo et al., 2006). Housefly and black soldier fly (BSF) maggots contain more than 20% crude fat and more than 35% crude proteins (Barroso, 2014, Makkar et al., 2014), making them a promising ingredient in feed formulation. A further aspect that has been mentioned for insect meals is their nutraceutical potential. For instance, BSF are very rich in lauric acid (C12) (Barroso 2014). Skrivanova et al. (2006) showed with an MIC test that lauric acid (C12) exhibits the highest activity against *Clostridium perfringens* compared with other medium chain fatty acids (MCFAs). Furthermore, lauric acid also has the lowest impact on favorable lactobacilli. These characteristics could help optimize performance and health by managing the microbiota in the upper part of the small intestine, which is dominated by gram-positive bacteria (Richards et al., 2005). Although these results were obtained with specific fatty acids (FAs) supplementation, the proposed effects can be mimicked with natural sources of FAs such as insect meals; however, further specific studies and investigations are urgently needed in this area to understand the potential gains and risks.

It has been observed that the nutritional value of insects is influenced by substrate composition. Specifically, fatty acid composition is one of the first observed changes in houseflies and black soldier flies in response to changes in substrate composition (Makkar et al., 2014; Hwangbo et al., 2009; Odesanya et al., 2011; Pretorius, 2011; Spranghers et al., 2015).

With regard to quality in animal products—and specifically the effects of dietary inclusion of BSF prepupae on fish fillet quality—Sealey et al. (2011) stated, '*BSF prepupae reared on dairy cattle manure and trout offal can be used to replace up to 50% of the fish meal portion of a practical trout diet for 8 weeks without significantly affecting fish growth or the sensory quality of rainbow trout fillets'*.

However, although scientific findings can help support and/or complete potential legal amendments concerning the use of insect meals in animal feed, the major barrier to the growth of the edible insect sector is the lack of precise and insect-inclusive legislation, standards, labeling and other regulatory instruments concerning the production, use and trade of insects in food and feed chains (FAO,2014). In fact, even though several projects (i.e., PROteINSECT) are still underway dealing with production of edible

insects for feed, they omit any focus on raising edible insects for food. One of the major limits to the full adoption of insects in feed formulation is the lack of robust and accurate analytical methods that can support legislation pertaining to these materials. From a practical point of view, insect meals and materials should actually be considered as PAP even though insects are not considered to be in that "family" in most related legislation.

Current legislation on insect material and detecting method

In the EU feed register, insects (Dried Insects 01586-EN) are defined as the dried whole or parts of insects and aquatic invertebrates in all their life stages of any species other than those that are pathogenic to humans and animals. This situation is complicated because according to EU Regulation 142/2011 (European Commission, 2011), 'processed animal protein' refers to animal protein derived entirely from Category 3 materials that were treated in accordance with Section 1 of Chapter II of Annex X (including blood meal and fish meal) to render them suitable for direct use as feed material or for any other use in feedstuffs, including pet food, or for use in organic fertilizers or for soil improvement; however, those regulations do not include blood products, milk, milk-based products, milk-derived products, colostrum, colostrum products, centrifuge or separator sludge, gelatin, hydrolyzed proteins and dicalcium phosphate, eggs and egg products (including eggshells), tricalcium phosphate and collagen. Thus, existing EU legislation does not prevent feeding farmed animals with live insects, but insect PAP may not be fed to farmed animals due to the feed ban restrictions. The most recent revision of the feed ban rules (European Commission, 2013b) has re-authorized processed animal proteins (PAPs) from non-ruminants for use as feed or feed ingredients in aquaculture since 1 June 2013, but that re-authorization has not changed the scenario. PAPs must still adhere to strict requirements to avoid any risk of cross-contamination with ruminant protein during collection, transport and processing. Under EC Regulation 56/2013 (European Commission, 2013a), category 3 PAPs (from non-ruminant species) would be permitted as feed only for aquacultured species.

Insects are not included in EC Regulation 56/2013 (European Commission, 2013a), mainly because the entire regulation focuses on slaughterhouse procedures and is

therefore not applicable. However, due to the increased interest in the topic, a modification of the annex is currently being drafted with the goal of including insects under EC Reg. 999/2001 (European Commission, 2001), but that has not yet been adopted. Furthermore, in the case of insects, a key issue is the substrate for their production in the mini-livestock: currently, 100% vegetable substrate and animal by-products (ABP) belonging to the category 3 (i.e., ABP derived from parts of animals that have been declared suitable for human consumption) may be used for growing insects. In accordance with this assumption, the use of ABP will limit the further use of insect meals in farm animal nutrition. The use of other substrates would require modification of the EU legislation on ABP.

Despite the above categorization and destination concerns about insect meals, a further step in defining future possible insect legislation is the implementation of analytical methods.

Currently, insect materials in the feed and food matrix have been considered as contaminants and/or extraneous matter. The standard method for determining insect fragments in food, such as flour and semolina, is acid hydrolysis. AOAC method 993.26 (1997) described also by Bhuvaneswari et al. (2011) and Perez-Mendoza et al. (2003) is a laborious and time consuming process that extracts insect fragments from flour by acid digestion and flotation. Insect fragments are generally brown in color and do not smash or shatter easily. Some of these hard-to-break fragments, such as mandibles, have clear edges and retain their characteristic shapes, making them easy to identify (Trematerra and Catalano, 2009). A few other methods that have sought to count insect fragments include using enzyme-linked immunosorbent assays (ELISA) (Quinn et al., 1992; Schatzki et al., 1993; Brader et al., 2002), DNA fingerprinting (Balasubramanian et al., 2007) and near-infrared spectroscopy (NIRS) (Perez-Mendoza et al., 2003). Hyperspectral imaging is a technique that provides spectral information about a scanned sample in a spatially resolved manner; each pixel has its own spectrum. The hyperspectral images can be described by a three dimensional array of size m n l, where m and n are the spatial dimensions (in detector size or pixels) in the x and y directions and l is the wavelength, or third dimension. Analytical tools from chemometrics combined with spatial image processing are used to reduce the dimensionality of hyperspectral data, extract the features, and then develop calibration models (Grahn and Geladi, 2007). A further approach was proposed by Bhuvaneswari et al. (2011), which compared speck counts using an electronic speck counter (SPX

Maztech MicroVision), acid hydrolysis and flotation (AOAC, 1997), and near-infrared (NIR) hyperspectral imaging in semolina seeded with insect fragments (50-300 fragments/50 g) of Tribolium castaneum (Coleoptera: Tenebrionidae). That study found a significant positive correlation between the number of insect fragments added and detected by all three methods; however, the goal in this case was also insect fragment detection as a measure of extraneous material.

Assuming that insect meals can be considered animal meals, and therefore as feed ingredients, existing techniques for PAPs can be considered as a robust starting point. The EU officially allows only two methods for the detection of animal proteins in feed: light microscopy and polymerase chain reaction (PCR). The latter supplies information on the species origin of the detected PAPs (European Commission, 2009a; European Commission, 2013c). Both methods have been validated for proper implementation of the feed ban. Light microscopy in combination with computer image analysis (IA) intended to identify bone particles or tissues in feeding stuffs has also been proposed (Ottoboni et al., 2014; Pinotti et. al., 2013). The findings in these studies have indicated that using microscopy in association with computer image analysis to identify the origin of PAPs appears promising, especially as a complementary method to DNA-based methods. Other methods applied in the feed sector include immunoassays and near-infrared microscopy (NIRM) (Tena et al., 2014). In light of this, it is important to improve our ability to characterize insect meals—not only in the case of pure material but also in practical conditions, such as in experiential feed formulation (pelleted and extruded). This goal can be achieved in various ways that may include methods already developed and applied to terrestrial PAPs. Existing methods should be adapted to insect materials when possible.

Risk profile related to use of insects in animal feed

Aware of the increasing interest in using insects in feed formulation, the Commission decided to ask the European Food Safety Authority (EFSA) to elaborate an initial scientific opinion on the microbiological, chemical and environmental safety risks arising from the consumption and production of insects as food and feed (EFSA, 2015). In this document, EFSA confirmed and compared results obtained by several

authors (Veldkamp et al., 2012; FAO, 2013; Makkar et al., 2014; Riddick, 2014; Sanchez-Muros et al., 2014; van Huis et al, 2015) stating that insects provide protein similar to soybean meal and fish meal and that insect products may partially replace traditional protein sources in animal feed.

The EFSA document noted a lack of knowledge concerning the occurrence of human and animal bacterial pathogens in farmed insects used as food and feed (EFSA, 2015). Scientific evidence reported in that EFSA scientific opinion (EFSA, 2015) suggests that, although pathogenic bacteria (such as Salmonella, Campylobacter and verotoxigenic *E. coli*) may be present in non-processed insects depending on the substrate used and the rearing conditions, the risk of transmission of these bacteria could be mitigated through effective processing steps applied between farming and consumption. Among different insects species that can be considered for micro-livestock, the most promising for industrial production in the western world are the black soldier fly (BSF) (*Hermetica illucens*), the common housefly (*Musca domestica*) and the yellow mealworm (*Tenebrio molitor*). These species have received increasing attention because they have the potential to valorize organic waste, which globally amounts to 1.3 billion tons per year. Black soldier flies, for instance, are naturally found on poultry, pig and cattle manure but can also occur on organic wastes such as coffee bean pulp, vegetables, carrion, and fish offal. This list introduces some potential substrates that can be used in different rearing systems; however, it also raises several safety implications that must be verified and checked, as suggested by the EFSA.

From another point of view, safety should be considered in combination with environmental issues. In this respect, the "insect potential" can be high (Leytem et al., 2009; de Haro Martí et al., 2010; Li et al., 2011). Livestock waste (urine and manure) contributes to environmental pollution (e.g., ammonia) that can lead to nitrification and soil acidification (Aarnink et al., 1995). In this sense, insect species such as the black soldier fly (*Hermetica illucens*), can be reared sustainably using such livestock waste as a substrate/material, thus reducing the nitrogen and phosphorus content (Leytem et al., 2010; de Haro Martí, 2010; Li, 2011). Alternatively, BSFs can not only be used to produce feed but also recycle waste into clean energy and reduce environmental pollution from manure. Nevertheless, according to current legislation (EC regulation 2009a and 2009b), larvae reared on manure cannot be used to feed animals. As an alternative, Li et al. (2011) proposed the use of insect fat (extracted grease) from *H. illucens* reared on manure for biodiesel production.

Water use is yet another issue because agriculture currently consumes approximately 70 percent of all freshwater used worldwide (Pimentel et al., 2004). Estimates of the volume of water required to raise an equivalent weight of edible insects are unavailable, but they could be considerably lower. In fact, several maggots (such as black soldier flies and common houseflies) do not require any additional water supply beyond the moisture of the substrate in which they are reared. Composting and stabilization of manure as well as the control of houseflies are also important in intensive animal production. The use of BSF larvae also reduces *Escherichia coli* counts in dairy manure (Liu et al., 2008), *Salmonella enterica* serovar enteritidis in chicken manure (Erickson et al., 2004) and reduces house fly populations in chicken manure (Sheppard and Craig, 1984).

The insect species selected, harvest stage, production methods, substrate and processing methods will all affect the occurrence and accumulation of contaminants in insect food and feed products. The greatest influence may be from the choice of substrate relative to the insect species reared on it (FASFC, 2014; ANSES, 2015; NVWA, 2014; Belluco et al., 2013; van der Spiegel et al., 2013). Unfortunately, data on the transfer of contaminants from different substrates to insects raised on them are extremely limited. The limited data available indicates that insects may accumulate heavy metals, in particular cadmium, from their substrates (Charlton et al., 2015 Diener et al. (2011) Banjo et al., 2010; Devkota and Schmidt, 2000). The presence of mycotoxins in farmed insects as well as the transference of mycotoxins from the substrate has been observed (Charlton et al., 2015; van Broekhoven, 2014). Moreover, mycotoxins may originate both from pathogenic fungi in the substrates and from mycotoxin production in the gut of insects (Schabel, 2010; FAO, 2013). Nevertheless, in spite of the available evidence, EFSA raised several uncertainties linked to the authorization of insects for feed and food due to the lack of knowledge in several areas such as prions and the extent to which insects act as mechanical vectors of them. Published data on hazardous chemicals in reared insects in the scientific literature are also scarce. Concerning the hazard of parasites, available information in the literature refers to non-European areas (mostly Asia) and to insects harvested in the wild. The risks of parasites from wild insects in the studied areas may be very different from the risks of parasites in farmed insects (EFSA, 2015). In addition, there is a lack of information about the exact details of insect processing and on the environmental impact of various mass-rearing insect production systems.

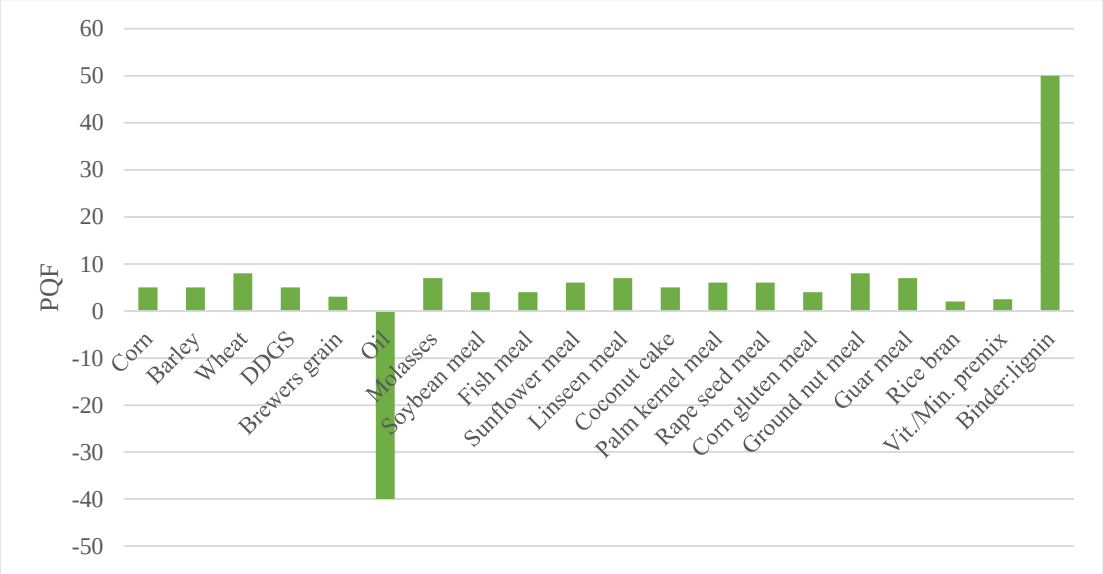
Feed technology

As already mentioned, the industrial compound feed sector is a key segment in the agri–food sector in general and in the chain of food products of animal origin in particular. In spite of that, producing high-quality safe feed and food products is first and foremost a question of good management practices at each stage of the feed and food chain (Pulina et al., 2014; FEFAC, 2014). Quality and safety of feed, however, are terms that not only refer to nutritional composition and values but also to specific physical and technological properties that can affect animal performance and food product quality. Specifically, beyond ingredients and nutrient features, compound feed quality and safety must be addressed in terms of physical properties, which are often linked to technical quality.

In the realm of technological quality, pelleting is a manufacturing process commonly used to densify and improve the handling characteristics and nutritive and economic value of granular materials (Theerarattananoon et al., 2011). Indeed, feed pelleting can be defined as the conversion of finely ground mash feed into dense, free-flowing pellets or capsules using a process that involves steam injection (moisture and heat) and mechanical pressure. There are several advantages in feeding farm animals with pelleted rather than mash feed, the most important of which is improved animal performance (improved feed intake, weight gain and feed conversion). Poultry fed on pelleted diets are less active, they 'sit' more and spend less time eating, resulting in lower maintenance energy requirements during eating and digestion compared to birds fed with mash feed (Nir et al., 1994). Other benefits of pelleted diets include increased feed density, reduced feed dustiness, wastage and selection, better handling of feed on mechanical feed lines and destruction of feed-borne pathogens. Additionally, pelleting improves microbial stability of the product (Čabarkapa et al., 2010). It was previously known that starch and its gelatinization are the most important factors for achieving a desired pellet quality (Wood, 1987; Thomas, 1996, 1997 and 1998); however, recent reports indicate that the positive impact of protein on pellet quality is important as well. Briggs et al. (1999) investigated this issue, observing that increasing the protein content also increased pellet durability. The same authors also reported that increasing the oil content above 7.5% greatly reduced pellet durability. This aspect has been described also by Farahat et al. (2015) who stated that oil's adverse effect on pellet quality is attributable to a coating effect on the feed particles that prevents steam

penetration. In addition, oil reduces the friction generated between die and feed particles causing a subsequent decrease in the starch gelatinization rate. As shown in Figure 6, oil has a pellet quality factor (PQF); values below 4.7 indicate poor pellet quality (Farahat, 2015).

FIGURE 6 PELLET QUALITY FACTOR (PQF) OF DIFFERENT FEED INGREDIENTS (FROM FARAHAT, 2015)



With regard to the moisture content, in general the optimum range for feed production is within 12 and 18% (see table 2). Moving above or under these referring value pellet quality decrease. Combining these factors with the potential use of insect material in animal feed formulation, it can be speculated that different insect materials (e.g. larvae meals vs insect protein extracts) can generate different problem in a feed plant, especially when pelleting is considered. In fact, some insect material sometimes is characterized by high moisture and fat content that can limit its pelletability.

TABLE 2 TYPICAL PROCESS PARAMETERS

Process	Temp. (°C)	Max. pressure (bar)	Moisture (%)	Max Fat (%)	Cook* (%)
Pellet press	60-100		12-18	12	15-35
Expander/ pelletpress	90-130	35-40	12-18	12	22-55
Dry extrusion	110-140	40-65	12-18	12**	60-90
Wet extrusion					
_Single-screw	80-140	15-30	15-35	22	80-100
_Twin-screw	60-160	15-40	10-45	27	80-100

*** % COOK IS STARCH GELATINIZATION MEASURED BY ENZYME SUSCEPTIBILITY. ** DRY EXTRUSION SUCCESSFULLY PROCESS FULL FAT SOY (18- 20% FAT AND OTHER INGREDIENTS WHERE FINAL PRODUCT DURABILITY IS NOT A CONCERN.**

In contrast to the pelleting process, extrusion is used mostly for pet food and fish feed production. In an extruder barrel, the material is exposed to thermal and mechanical treatments, plasticizing and shaping the material. The quality of the extruded product is influenced by various factors including die geometry, feed composition, feed moisture, particle size, feed rate, screw configuration, screw speed, etc. and specific combinations of these factors shape the final product's characteristics. Unlike the pelleting process, the extrusion process can control the density of a product, resulting in high digestibility and improved physical characteristics (Guy, 2000). Gelatinization is the major transition of starch during thermal processes. When the mash is extruded through the die, the release of pressure and heat causes the starch to expand and gelatinize (Shankar and Bandyopadyay, 2005). It is recognized that extrusion has dramatic effects on starch chemistry compared to less aggressive feed processing techniques such as steam-pelleting or screw-pressing. Gelatinization and expansion of the starch also increases its nutritional value by increasing its digestibility (Bergot and Breque, 1983; Jeong et al., 1991; Glencross, 2011). Another benefit of the extrusion process is its denaturing effect on some of the anti-nutritional factors in raw materials such as protease inhibitors and lectins, which are affected by the heat treatment (Refstie et al., 1998; Francis et al., 2001). With regard to the effects of feed formulas on extrusion performance, several authors (Madeleine et al., 1979; Schweizer et al.,

1986) reported that adding lipids during extrusion generally retards the degree of gelatinization and affects dough rheology in the barrel, which affects the dough flow properties (Hsieh et al., 1991) and thus the degree of starch gelatinization of the feed materials (Malkki et al., 1984). Unlike pelleted feed, extruded feed can include higher amounts of both fat and water. For example, twin-screw extruders can handle viscous, oily, sticky or wet material, including products with greater than 25% of internal fat. Using this extrusion technology also makes it possible to add high levels of wet ingredients (i.e., fresh meat) up to 35% (Guy, 2000). These features make the extrusion process in general and twin screw extrusion technology in particular the most popular for high-fat aquafeed (i.e., salmonid) and super premium pet food (with fresh meat) production (Guy, 2000).

Matching recent advances in nutrition science with feed industries' innovation needs

Worldwide, the consolidation and intensification of the feed industry has resulted in more tons produced from fewer feed mills. In the European Union between 2005 and 2010, feed mill size has increased from approximately 10,000 tons to 50,000 tons per feed mill per year, while the number of feed mills decreased by 80%. This trend is mirrored in the US and even in China, where the number of feed mills has dropped from over 15,000 to 10,000. The industrialization of the feed industry has resulted in an increased specialization and efficiency of manufacturers and suppliers (Connelly, 2013).

A further feature of the feed industry today is its competitiveness. In this respect, feed cost is determined by four components: the cost of raw materials (approximately 70% of the overall cost of feed), labor costs, energy price, and depreciation of milling facilities. Accordingly, feed companies are intensifying their commitment to innovation, which is considered the key to sustainable food security. Through innovation, the feed industry can improve resource-efficiency, adapt to trade changes, and improve food safety, diversity and quality while maintaining the competitiveness of the agri-food sector and creating more and better jobs in rural areas (Hogan, 2015). With respect to the European situation presented above, it appears clear that the livestock sector in general and the feed sector in particular need to take into account

several new challenges including environmental impact, the scarcity of raw materials, and societal acceptance. A common denominator among many of these issues, which are often politically sensitive, is not only sustainability but also innovation (Geraldine, 2014). Indeed, accelerated research and technology development—based on an innovative approach—will be crucial for developing feed solutions able to guarantee the EU livestock sector and remain competitive and sustainable in the global market (EUFETEC, 2013).

Several of these issues are linked with the "insect story." As reported earlier and also elsewhere (Insects to Feed the World Conference, 2014), the potential of insects for human food and animal feed is highly relevant in view of their good nutritional quality; human population growth, and corresponding higher demands for animal proteins in the form of meat and fish the fast rising costs and quantities of major protein sources needed to feed the growing number of farmed animals, and the serious environmental impact of our current high meat consumption food habits and animal farming practices, which use feed grains that could be directly consumed by humans (Makkar, 2014; Barroso, 2014; van Huis 2013; Veldkamp et al. 2012; Sánchez-Muros et al., 2014; Rumpold and Schlüter, 2013). All these concerns must be combined with feed industry needs because those represent the interface with the livestock system. However, because insect inclusion in animal feeds should be considered as a new approach, its acceptance as an innovation and as good practice must be verified.

Aim

The general aim of this study was to investigate new technologies in feed formulation and production. Several aspects of the field of feed production were investigated to evaluate innovative solutions in the supply of protein feed ingredients. Accordingly, three main sub aims were identified:

1. Evaluation of new ingredients in feed formulation and the impact of technology on nutritional value.

   The approach to test the hypothesis was:

   - Investigate the inclusion of insect material (*Hermetia illucens*) as is in an experimental extruded feed.
   - Evaluate the impact of extrusion on the nutritional value and digestibility of experimental mixtures containing insect material (*Hermetia illucens*).

2. Implement the official methods for the detection of PAPs i.e., microscopy in combination with image analysis measurements.

   The approach to test the hypothesis was:

   - Evaluate microscopy in combination with image analysis measurements as an additional tool (in combination with classical microscopy) for:

     Species-specific identification of bovine and swine bone containing material (Experiment 1).

     Characterization of fish bone lacunae in aquafeed-extracted material (Experiment 2).

   - Evaluate an analytical approach for tracing insect material in feed. Specifically, preliminary experiments were performed to characterize insect originated pure material (i.e., insect meal) using microscopy (Experiment 3).

3. Investigate potential areas for innovation in research and development in the feed sectors (i.e., use of new or innovative raw materials); identify the key topics of research and development in the feed sector; obtain stakeholders' opinions on how to integrate the most valuable identified factors into practice. *The approach to test the hypothesis* was:
    - Perform a survey study addressed to European feed companies in two countries (Italy and Serbia). The questionnaire was based on three main sections: i) Company Overview (CO); ii) Products and Process Features (P&P); iii) Research, Development and Innovation (R&D).

Material and method

Evaluation of new ingredients in feed formulation and impact of technology on nutritional value

Evaluation of inclusion of insect material (*Hermetia illucens*) as is in an experimental extruded feed

Mixture formulation

Wheat baking flour obtained from the experimental baking lab of the Department of Applied Bioscience of Ghent University, and BSF larvae and prepupae were the main ingredients used in this study. The larvae were sourced from a private company that produces BSF for experimental use (Antwerp, BE), while the prepupae were supplied by the Department of Crop Protection of the Faculty of Bioscience Engineering at the University of Ghent. Sunflower oil was obtained from a local supermarket. Premixes of wheat flour and either BSF larvae or prepupae were formulated in the ratio 75:25, respectively, and contained approximately 10.93 to 11.48 % protein (wet basis) and from 23.65 to 24.2 % water. These premixes of flour and raw insects were prepared in a typical household blender. Sunflower oil was added to the prepupae premix to obtain mixtures ranging from 3.15 to 5.37% fat. The details of these mixtures are summarized in table 6.

TABLE 3 CHEMICAL COMPOSITION OF AQUAFEED USED IN THE EXPERIMENT 1

	Moisture %	P % on *wet basis*	CF% on *wet basis*	Ash % on *wet basis*
Prepupae + wheat 25:75 (NO oil)	24.21	11.48	3.15	2.34
Prepupae + wheat 25:75 (+oil 1)	24.02	11.39	3.89	2.32
Prepupae + wheat 25:75 (+oil 2)	23.84	11.30	4.63	2.30
Prepupae + wheat 25:75 (+oil 3)	23.65	11.21	5.37	2.29
Larvae + wheat 25:75 (NO oil)	23.71	10.93	4.62	1.67

P – PROTEIN; CF – CRUDE FAT

Extrusion

Extrusion was performed on a co-rotating, conical twin-screw mini extruder (HAAKE™ MiniLab II) (figure 7). The barrel was composed of a single controlled temperature zone and heated by an electric cartridge heating system (air-cooled). The barrel can be split horizontally and opened to enable rapid removal and cleaning of the barrel and the screws. The mini extruder was manually fed using a lab spoon, and a pestle was used to force the raw materials into the extruder. The end of the extruder was equipped with a single circular die opening 2 mm in diameter.

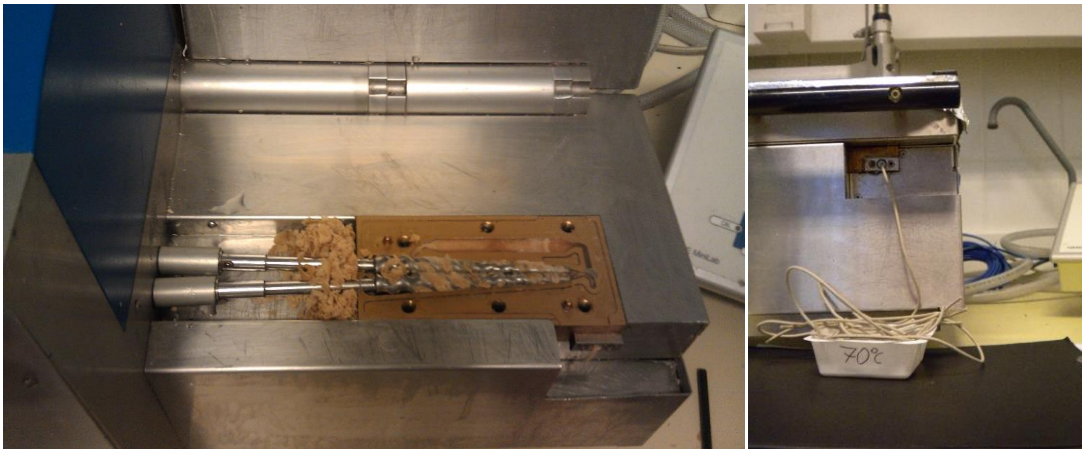
Extruded material was collected when die flow and torque value were both stable for at least 2 min, and then cooled at room temperature and packed in plastic bags.

The variables studied were the level of fat used in premix, extruder screw speed and barrel temperature.

The extrusion test was split in two part:

Part 1: The experimental design consisted of four fat percentages (3.15, 3.89, 4.63 and 5.37 %), a single barrel temperature (60°C) and a single screw speed (60 rpm). Premix containing larvae material without added fat was compared to premix containing prepupae material in the same ratio of flour to insect with increasing amounts of oil (3.15 to 5.37 tot. fat %).

FIGURE 7 CO-ROTATING, CONICAL TWIN-SCREW MINI EXTRUDER (HAAKE™ MINILAB II). DETAIL OF THE BARREL OPENED TO REMOVAL AND CLEAN OF THE BARREL AND THE SCREWS (ON THE LEFT) AND EXTRUDER WORKING AT 70°C.



The torque value was recorded for each tested mixture; mixtures with value <100 Ncm were considered extrudable and a decrease in this value in a mixture indicates higher extrudability.

Part 2: A single mixture was used to evaluate the effect of barrel temperatures on nutritional value and digestibility. This experiment used a single screw speed (100 rpm) and four different barrel-temperatures (60, 70, 80 and 90°C) (figure 7, right side).

Evaluation of the impact of extrusion on the nutritional value and digestibility of experimental mixtures containing insect material (*Hermetia illucens*).

In-vitro digestibility test

In vitro digestibility assays were performed on freeze dried prepupae material and extruded experimental feed as is. Two different in vitro protocols (table 4) were adopted for dry matter/organic matter and protein digestibility, respectively, as described by Dierick (1991). According to the protocol, varying amounts of sample were finely ground with a mortar and pestle (< 1 mm), weighed to an accuracy of ± 0.1 mg and incubated in incubation flasks (figure 8). Analyses were performed in triplicate.

FIGURE 8 IN VITRO DIGESTIBILITY TEST: INCUBATION FLASKS IN SHAKING WATER BATH (ON THE LEFT) AND INCUBATION FLASK AFTER DOUBLE INCUBATION WITH PEPSIN AND PANCREATIN

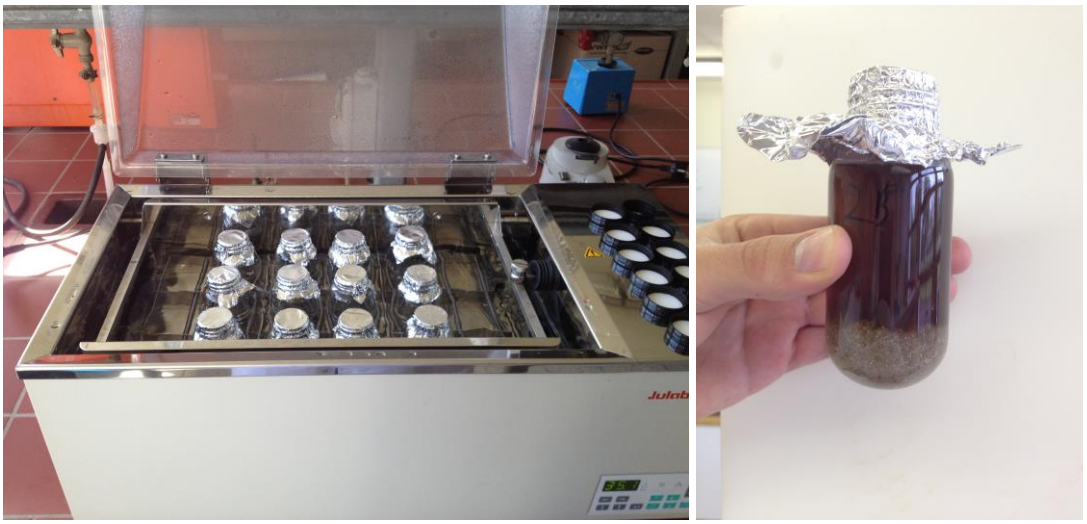


TABLE 4 IN VITRO DIGESTION PROTOCOL (MODIFIED FROM DIERICK ET AL. 1991)

	Protein	Dry matter/Organic matter
Step 1	150 mg protein Incubation with pepsin 4h 37° C	2 g sample Incubation with pepsin 4h 37° C
Step 2	Incubation with pancreatin 4h 37° C	Incubation with pancreatin 4h 37° C
Step 3	+ Phosphotugstenic acid (PTA) Centrifugation residue: undigested protein Supernatant: α amino-group det.	Centrifugation residue: undigested DM

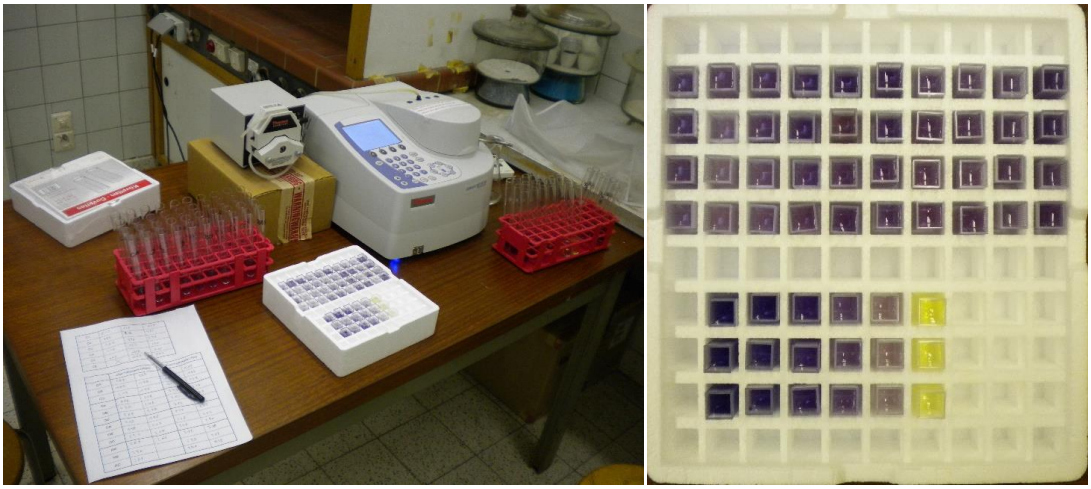
After two washings, followed by centrifugation, with 10 ml of Phosphotugstenic acid (PTA) and 5 ml of PTA + 20 ml of distilled water, respectively, the undigested residues were transferred to Kjeldahl tubes and dried at 70°C overnight. Undigested nitrogen was measured by the Kjeldahl [ISO method 5983-1 (2005)]. The in vitro digestibility of protein was calculated from the difference between the nitrogen in the original sample and the undigested residue.

Supernatants were filtered with filter paper, and free amino acids (AAs) and peptides concentrations were determined according to the α amino-group.

After the pepsin and pancreatin incubation in the dry matter/organic matter digestibility test and three consecutive centrifugation and washings with 2x 20 ml distilled water, the undigested residues were dried at 105°C overnight. The in vitro digestibility of dry matter was calculated from the difference between dry matter in the sample and the undigested residue. Residue was incinerated in a muffle oven for 4 h at 550°C, and OM digestibility was then calculated from the difference between dry matter and the ash in the residue.

α-Amino nitrogen (αNH2–N) was determined as an index of total free amino acids from supernatant samples obtained after protein digestion (figure 9). The supernatants were filtered with filter paper and then diluted 20x with distilled water. Samples were tested in duplicate using the methodology described by Oddy (1974). Absorbance was measured at 570 nm using a spectrophotometer (Thermo SCIENTIFIC, Model GENESIS 105 UV-VIS).

FIGURE 9 DETAILS OF α AMINO NITROGEN (αNH2–N) DETERMINATION IN SUPERNATANT



Di- tri- and tetra peptides are also contained in the supernatants (Dierick, 1991). To determine the total AAs, 1 ml of supernatant of each sample was hydrolyzed with 1 ml of HCl 12 M 24 h at 100°. The samples were successively cooled at room temperature, and then 2 ml of NaOH 6 M were added to neutralize the solution. The total amount of AAs was determined after 20x dilution with distilled water of the hydrolyzed supernatant as previously described.

Di- tri- and tetra peptides were calculated by determining the difference between AAs before and after hydrolysis.

Implementation of the official methods for the detection of Processed Animal Proteins (PAPs) in feed

In order to improve the official method for detect constituents of animal origin in feedstuff tree main experiment have been performed.

Experiment-1 Evaluation of microscopy in combination Image analysis for MBM characterization: comparison between bovine and swine material

The official analytical method were combine with image analysis measurements, in order to discriminate between bovine and swine lacunae. This experiment has been done in the frame of Progetto di Ricerca Corrente 2010-2013, Id. IZSPLV 12/10/RC title "Identificazione di specie delle proteine animali trasformate nei mangimi: sviluppo e confronto di tecniche microscopiche e immunoistochimiche" coordinated by the National reference laboratory for feed and feed additives (C.Re.A.A IZS Torino).

For this study, 10 samples of controlled origin and processing were used, containing bovine (BOV, 5 samples) or swine (SUS, 5 samples) meat and bone meal (Walloon Agricultural Research Centre - CRA-W, Belgium; VESPA, University of Milan). In each experiment, the samples were analyzed using the microscopic method (European Commission, 2013a). Sediment fractions of each sample were observed with a compound microscope (Olympus BX41, Germany) at several magnifications, in order to obtain several bone fragment lacunae images at X40 for each sample.

Using a digital camera and image analysis software (Image-Pro Plus 7.0, Media Cybernetics Inc., Silver Springs, USA), 362 bone fragment lacunae images at X40 were obtained. Images were acquired according to Pinotti (2009). The images were then processed in order to obtain a monochrome mask for each lacuna (Figure 17). On each lacuna, 30 geometric variables were measured as previously described (Pinotti et al., 2013). Using this method, size descriptors and derived shape descriptors can be identified. The size descriptors, such as area, perimeter, axis minor and major, radius min and max, etc. (Table 5) represent direct measurements on bone lacunae, and are also termed as dimension (Primary) descriptors. On the other hand, the derived shape

parameters (Table 6) are constructed by combining the various size parameters so that the dimension units are cancelled out (Russ, 2005). Derived shape descriptors are represented by V2, V3, V4, V20, V21, V34, V55, V56 and V58. All lacunae measurement data were collected in Excel files and used for dataset assembly. Tables 5 and 6 report the full list and description of all the 30 geometric variables used.

TABLE 5 SIZE / PRIMARY DESCRIPTORS

ID	Variable	Unit	Description
V1	Area	μm^2	Area of the object, includes area of the hole if 'Fill Holes' is turned on
V11	Axis major	μm	Length of major axis of ellipse
V12	Axis minor	μm	Length of minor axis of ellipse
V13	Diameter max	μm	Length of longest line joining two points of the object's outline and passing through the centroid
V14	Diameter min	μm	Length of shortest line joining two points of the object's outline and passing through the centroid
V15	Diameter mean	μm	Average length of diameters measured at 2 degree intervals and passing through the object's centroid
V16	Radius max	μm	Maximum distance between object's centroid and outline
V17	Radius min	μm	Minimum distance between object's centroid and outline
V19	Perimeter	μm	Length of the object's outline. More accurate than previous version. Old version now called perimeter2
V28	Size (length)	μm	Feret diameter (i.e. caliper length) along major axis of object
V29	Size (width)	μm	Feret diameter (i.e. caliper length) along minor axis of object
V30	Perimeter 2	μm	Chain code length of the outline. Also includes any outlines of holes. Faster but less accurate than perimeter
V32	Perimeter (convex)	μm	Perimeter of the convex outline of the object
V33	Perimeter (ellipse)	μm	Perimeter of the equivalent ellipse
V35	Polygon area	μm^2	Area included in the polygon defining the object's outline. Same polygon as that used for perimeter
V40	Box Width	μm	Width of the object's bounding box
V41	Box Height	μm	Height of the object's bounding box
V42	Min feret	μm	Smallest caliper (feret) length
V43	Max feret	μm	Longest caliper (feret) length
V44	Feret mean	μm	Average caliper (feret) length
V57	Convex Area	μm^2	Area of a polygon which has major axis and minimum axis for sides

TABLE 6 DERIVED SHAPE DESCRIPTORS

ID	Variable	Description
V2	Aspect	Ratio between major axis and minor axis of the ellipse equivalent to object
V3	Area/Box	Ratio between area of object and area of its bounding box
V4	Box X/Y	Ratio between width and height of object's bounding box
V20	Radius ratio	Ratio between max radius and min radius
V21	Roundness	(perimeter2)/(4πarea). Uses 'perimeter2' and 'area' by default. Select 'perimeter' and 'area' for more accurate roundness
V34	Perimeter ratio	Ratio of convex perimeter to perimeter
V55	Form factor	4πArea/Perimeter2
V56	Roundness 2	4Area/πAxis major2
V58	Solidity	Area/Convex Area

Bovine and swine lacunae measurements were analyzed using one-way analysis of variance (one-way ANOVA) in order to compare means of the two species (GLM procedure of SAS statistical software 9.3). The analysis has been performed using the following model:

$y_{ij} = \mu_j + \varepsilon_{ij}$

Where y_{ij} are the observations (measurements), μj is the mean of the observations for the jth group (specie) and ε_{ij} is the random error. Differences with P values 0.001 were considered significant. Furthermore, since considerable overlap of the species distributions of the sizes of individual lacunae was expected (Pinotti et al., 2013), graphic test (box-plot) for mean and median comparisons has been done. Accordingly, the BOXPLOT procedure was performed in order to displays the mean, median, quartiles, minimum and maximum observations and outliers for each single species.

Experiment-2 Characterization fish material and comparison with land animal material

Part A In the same field, a different experiment was performed that investigated the use of microscopy in combination with image analysis measurements for the characterization of fish bone lacunae in aquafeed-extracted material. For this experiment, 6 samples of commercial compound fish feeds containing fish meal were used (proximate analyses are reported in table 7).

TABLE 7 CHEMICAL COMPOSITION OF AQUAFEED USED IN THE EXPERIMENT 2

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
DM (g/kg)	94.28	95.5	93.54	94.1	94.59	92.53
CP (g/kg DM)	45.82	42.89	44.42	45.45	42.64	48.82
CF (g/kg DM)	20.64	19.99	16.49	23.25	21.97	18.51
aNDFom (g/kg DM)	32.28	32.14	43.14	29.07	25.08	35.74
Ash (g/kg DM)	9.15	9.32	6.4	5.15	6.58	7.19

DM = DRY MATTER; CP = CRUDE PROTEIN; CF = CRUDE FAT; ANDFOM, NEUTRAL DETERGENT FIBER ASSAYED WITH A HEAT STABLE AMYLASE AND EXPRESSED EXCLUSIVE OF RESIDUAL ASH.

The samples were analyzed using the microscopic method. Bone fragment lacunae images were acquired and processed using the same protocols as Experiment 1 and all lacunae measurements were collected in Excel files and used for dataset assembly.

Part B The results obtained in Part A (fish bone lacunae present in commercial aquafeed materials) were merged with raw data obtained from authentic samples of poultry and mammals, used in Pinotti et al. (2013). Specifically, measurements obtained from 1081 bone lacunae (644 from mammals and 437 from poultry), acquired from 14 mammalian and 7 poultry samples were merged with the aquafeed dataset (258 bone lacunae).

Statistical analysis

Part A As in experiment 1, aquafeed 1 to 6 lacunae measurements were analyzed using one-way analysis of variance (one-way ANOVA) to compare means of the 6 samples (GLM procedure of SAS statistical software 9.3). Furthermore, to compare the size of lacunae and evaluate the overlap of the six within-sample distributions,

boxplots of these distributions were examined. Accordingly, the BOXPLOT procedure (PROCBOXPLOT) was performed to displays the mean, median, quartiles, minimum and maximum observations and outliers for each single sample.

Part B Data obtained from fish material (258 lacunae) in aquafeed were compared with the lacunae from poultry and mammals (644 from mammals and 437 from poultry) using the morphometric descriptors reported in Pinotti et al. (2013). The analysis was performed following the same model used in experiment 1. The BOXPLOT procedure (PROCBOXPLOT procedure of SAS statistical software 9.3) was also performed.

Experiment-3 Characterization of insect material by microscopy and comparison with marine organism material.

Evaluation of an analytical approach for tracing insect material in feed.

Starting from the assumption that insect material is considered of anomalous origin, a small-scale study in collaboration with the European reference laboratory for animal proteins (EURL-AP) was launched. Specifically, the microscopy method was tested for its suitability in characterizing insect materials.

This experiment used 1 sample of pure insect meal material and 1 sample of marine organism meal consisting of *Hermetia illucens* larvae and shrimp, respectively. Shrimp material was selected because previous experience (Veys, personal communication) indicated some similarities between insect (terrestrial arthropods) and marine arthropods. Dried pure samples (EURL-AP) were ground with a mortar and pestle. Subsequently, microscopic slides were prepared using Norland Optical adhesive 65 as the embedding agent. After drying, each sample was examined using a compound microscope at several magnifications. Depending on the image quality, insect/marine organism fragment images were acquired at 10X, 20X or 40X using a digital camera.

Three staining reagents were also evaluated and used to enhance fragment identification.

Alizarin red staining

EURL-AP (2013) in the Standard Operating Procedure concerning the Use of staining reagents reports that alizarin red stains bones, fish-bones and fish scales along a range from bright red to pink. For this reason, alizarin red is authorized as a staining reagent in Reg. EU 51/2013 (European Commission, 2013c) concerning methods of analysis for the determination of constituents of animal origin for the official control of feed. Nevertheless, alizarin red is not specific to bone; it colors bone's major mineral constituent, hydroxyapatite. It is also reported to react with calcium phosphates (e.g., tricalcium phosphate) (EURL-AP, 2013). Shrimp exoskeleton is naturally rich in calcium (Watkins et al., 1982) and small amount of calcium is present in *Hermetia illucens* larvae (Finke et al., 2013). In light of this, alizarin red staining was tested for efficacy on both insect and marine material using the following staining protocol.

Alizarin red stain Color Index Number 58005, Sigma-Aldrich 3050 Spruce Street, Saint Louis, MO 63103, USA was used.

A dilute solution consisting of 100 ml water and 2.5 ml 1 M hydrochloric acid was prepared, and 200 mg Alizarin red were added to this solution.

100 mg of dry sample were transferred into a glass test tube and rinsed twice with approximately 5 ml ethanol (each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). Before using the staining reagent, the sample was bleached by adding at least 1 ml sodium hypochlorite solution. The reaction was allowed continue for 10 minutes. The tube was filled with water, the sample was allowed to settle for 2-3 minutes, and then the water and any suspended particles were poured off. The sample was rinsed twice more with approximately 10 ml of water (each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). Two to ten or more drops (depending on the amount of residue) of the Alizarin red solution was added. The mixture was shaken and the reaction was let occurred a few seconds. The colored sediment was rinsed twice with approximately 5 ml ethanol followed by one rinse with acetone (each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). The sample was placed in an oven at 68°C until completely dry.

Chlorazol Black staining

Chlorazol black is a stain with a high affinity for chitin, a unique structural polysaccharide (a homopolymer of β-[1,4]-linked D-N-acetylglucosamine (Thomas et al., 2008). As reported by Finke (2009) *Hermetia illucens* is rich in chitin. This structural polysaccharide in combination with calcium is also one of the main components in shrimp exoskeletons (Watkins et al., 1982; Sagheer et al., 2009). For these reasons, chlorazol black stain (Color Index Number 30235, Sigma-Aldrich 3050 Spruce Street, Saint Louis, MO 63103, USA) was tested for both insect and marine material using the following staining protocol.

Dry samples (100 mg) were transferred into a glass test tube and rinsed twice with approximately 5 ml ethanol (each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). Before using this staining reagent, the sample was bleached by adding at least 1 ml sodium hypochlorite solution. The reaction was allowed to continue for 10 minutes. Next, the tube was filled with water, the sample was let settle 2-3 minutes, and the water and any suspended particles were poured off. The sample was rinsed twice more with approximately 10 ml of water

(each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). The sample was rinsed once with approximately 5 ml acetone, vortexed and decanted, let settled, and then the acetone was poured off. A few drops (depending on the amount of residue) of the chlorazol black solution were added. The mixture was shaken and the reaction was let occur a few seconds. The colored sediment was rinsed twice with approximately 5 ml ethanol followed by three rinses with acetone (each time a vortex was used; the solvent was allowed to settle approximately one minute and then poured off). The sample was placed in an oven at 68°C until completely dry.

Aniline blue staining

(1→3)-β-D-glucans specifically bind to the triphenylmethane dye −Aaniline blue− (Nakanishi et al., 1974).

1% (w/v) aniline blue stock solution was made by dissolving water soluble aniline blue (color index 42755 Sigma-Aldrich 3050 Spruce Street, Saint Louis, MO 63103, USA) in deionized water.

Staining procedure

A few drops (depending on the amount of residue) of the 1% aniline blue solution were deposited directly on the microscopic slides as embedding agent. Microscopic slides were mounted using adequate mounting medium and covered with coverslips in accordance with the SOP established by the EURL-AP and published on its website. Each coverslip was sealed around its edges with melted VALAP as described below.

To prevent drying of the embedding aniline solution during long-term imaging, each coverslip was sealed with VALAP. VALAP is a 1:1:1 mixture of Vaseline, lanolin, and paraffin (beeswax can be substituted for paraffin) prepared by melting and mixing these ingredients at very low heat. After melting and mixing, the VALAP can be poured into small containers and stored at room temperature for future use. After placing the coverslips on the top of the mounting aniline solution, the slide was sealed by heating a metal spatula over a flame, dipping the spatula tip into the VALAP to melt and pick up some VALAP, and then spreading the melted VALAP around the four sides of the coverslip, effectively sealing the agar pad and preventing drying. At this point, the insect or marine material was ready to be imaged using a microscope to perform long-term analysis (hours).

Aniline blue solution is frequently used for vegetable structure stains. For example, Schenk and Schikora (2015) quantified callose depositions with a fluorescence microscope using a DAPI filter. They reported that the optimal excitation wavelength for aniline blue is 370 nm, and the emission maximum is by 509 nm. Similar results were reported by Smith and McCully (1978); the fluorochrome fluoresces weakly with a maximum emission approximately 455 nm, but the fluorescence shifts to longer wavelengths (500–506 nm) when complexed with isolated β-1,3-glucans, cellulose or mixed-linked glucans. Thus, the same wavelength ranges were adopted in this study for insect examination.

Next, each sample was examined under UV light using a compound microscope at several magnifications. Depending on the image quality, insect/marine organism fragment images were acquired at 10X, 20X or 40X using a digital camera. We used the Zeiss AxioVision 3.0 software (at the EURL_AP).

Survey study

Investigation of potential areas for Innovation in Research and Development in the feed sectors

The survey was conducted from July to October of 2014. A questionnaire containing 29 questions (Figure 1) was developed and sent to 113 feed companies consisting of Italian and Serbian stakeholders in the feed sector, and in particular extra small, small, medium and multinational companies (referred to later as XS, S, M, L). The questionnaire was sent to all relevant companies in the Italian and Serbian regional trade association databases. The questionnaire included three main sections: i) Company Overview (CO); ii) Products and Process Features (P&P); iii) Research, Development and Innovation (R&D). The elements for each dimension (CO, P&P and R&D) and their corresponding questions were selected using contributions from in-house experts in feed technology, feed and animal nutrition, and economic science. Briefly, as reported in Table 1, CO included general information such as year of foundation, company size and owner profile. The P&P section covered types of feed production (monogastrics or ruminants), production plant machinery, list of feed additives used, and so forth. Finally, R&D included areas of innovation, perceived difficulties (e.g., economic and bureaucracy), projects in the past and present, and consistency of a budget dedicated to R&D.

Statistics

The recorded data were analyzed using two different approaches: descriptive statistics and simple correspondence analysis. Specifically, data collected by open response and multiple-choice answers in each section (CO, P&P and R&D), were processed to obtain frequency statistics and used to create graphs of the data. Furthermore, for a specific set of questions, a simple correspondence analysis was performed. In this trial, companies were grouped according to their size (see below) and type of production (feed for monogastric, ruminant, or both) to highlight the relationships between these features and the areas of innovation in R&D (source/type of raw materials, product design, industrial process, packaging, marketing, nutritional content of the product, company organization) in which they had been most innovative in the last 3 years. Companies were classified into four categories: extra small (XS—less than 20 employees), small (S—21 to 50 employees), medium (M—51 to 100 employees) or large (L—more than 100 employees). Companies were also classified into four

categories by type of feed production: monogastric, ruminant, both, or no-answer. A simple correspondence analysis with a symmetric normalization model (Beh, 2004; Hoffman and Franke, 1986; Lebart et al., 1984) was performed using SPSS 22.0 statistical software. This multivariate statistical method is suitable for exploring relationships between items of two nominal variables. Accordingly, in the present study, the correspondence analysis considered the company dimension and the type of feed production (monogastric vs. ruminant) for each feed plant and the areas in which they had been most innovative in the last 3 years. Differences or similarities can be interpreted by looking at the position of points in a Cartesian plane, called a biplot. Briefly, the closer the points are in the plots, the more similar they are considered. In fact, statistically they are close because they contribute to the constructions of the same dimension of the graphs. As explained in Gaviglio et al. (2014 and 2015), the results are evaluated by taking inertia, mass, contribution to dimension, squared correlation and quality of each point into account. The inertia of a dimension represents the eigenvalue and reflects the relative importance of each dimension of the biplot. The mass measures the frequency of each pair of variables in the interviewees' answers. The contribution to dimension indicates the importance of each point to the dimension considered. The coordinates of the point, by definition, are the distances of each point from the origin of the plot and indirectly indicate whether the considered variables are significantly correlated with each other. Finally, squared correlation approximates the accuracy of a point in constructing the axis, while quality approximates the accuracy of a point considering the whole biplot.

Results and discussion

Evaluation of new ingredients in feed formulation and impact of technology on nutritional value

Evaluation of inclusion of insect material (*Hermetia illucens*) as is in an experimental extruded feed

The two lowest fat mixtures were considered not extrudable. By increasing the fat content from 3.9 to 4.6 %, the torque value decreased significantly to an acceptable level for extrusion (Table 8). The best extrusion performance was obtained with the highest fat mixtures (5.4%). The lubricating effect of fat inside the extruder probably reduced the friction between the dough and the screw elements and between the dough and the barrel, resulting in a decrease of torque value. For the mixtures with identical fat content, those containing 30% of total fat from added oil exhibited considerably higher torque values. Although this aspect of feed extrusion should be deeply investigated, an effect from the lipid source could be conjectured. For instance, Lin et al. (1997) observed that fat sources could affect the degree of gelatinization in extruded pet food production.

TABLE 8 EFFECTS OF FAT ON EXTRUSION PERFORMANCES ON TESTED MIXTURES

Mixture	Ratio	Fat %	Torque value Ncm	Extrudability
Prepupae+wheat	25:75	3.2	200-400	Not extrudable
Prepupae+wheat	25:75 + oil 1	3.9	>400	Not extrudable
Prepupae+wheat	25:75 + oil 2	4.6	100-130	Acceptable
Prepupae+wheat	25:75 + oil 3	5.4	50-100	Acceptable
Larvae+wheat	25:75	4.6	80-120	Best Value

Evaluation of the impact of extrusion on the nutritional value and digestibility of experimental mixtures containing insect material (*Hermetia illucens*).

In-vitro digestibility tests

No differences were observed in torque value (which ranged between 80-120 Ncm) between different barrel temperatures; thus, the mixture used was considered efficiently extrudable with a screw speed of 100 rpm when the temperature range was between 60 and 90°C.

With regard to water loss, the highest value was recorded after extrusion at 60°C. Unexpectedly, water loss decreased as the barrel temperature increased (Table 9). Observing the extruded material, the surface became smoother and brighter as the barrel temperature increased, indicating a higher degree of gelatinization. Chiang and Johnson (1977) confirm this hypothesis: they reported that gelatinization increases from 35% to 95% when the temperature moves from 65 to 110°C in wheat flour mixtures containing 24 % water. The formation of an external layer probably prevented the water loss. By contrast, the material extruded at 60°C presented a coarse and rough surface, confirming that surface gelatinization was lower; thus, water loss was not prevented in this case.

TABLE 9 EFFECT OF TEMPERATURE BARREL ON WATER LOSS IN EXTRUDED MIXTURES

Extr. T°	DM	water loss %
Control	75.21	-
60	80.23	20.22
70	79.68	18.03
80	79.50	17.31
90	78.94	15.04

EXTR. T° - EXTRUSION TEMPERATURE; DM – DRY MATTER

In vitro digestibility extruded mixtures

The extrusion process slightly increased the protein digestibility. The highest increases (1.05 %) in protein digestibility were recorded when extrusion was performed at 60 and 70°C, although the differences were not significant (Table 10).

TABLE 10 EFFECT OF BARREL TEMPERATURE IN EXTRUDED MIXTURES ON IN-VITRO PROTEIN DIGESTIBILITY

	Rep	P dig. %	
	N°	Mean	CV %
CTR	3	93.54	0.63
60	3	94.49	0.91
70	3	94.54	0.31
80	3	94.21	0.15
90	3	94.02	0.63

P DIG. – PROTEIN DIGESTIBILITY; CV – COEFFICIENT OF VARIABILITY

Total AA in supernatants reflected the results obtained for P digestibility calculated from the difference between nitrogen in the sample and the undigested residue. Free AA values were higher in 60°C extruded mixtures than in controls and tended to decrease as the barrel temperature increased to 90°C (Table 11). Boye et al. 1997 reported that at temperatures above 80°C, there is a loss of almost all secondary and tertiary structures. Initially, there is a reversible unfolding step, followed by irreversible alterations, which include aggregation (intermolecular interactions), scrambling of disulfide bonds, and chemical modifications of side-chains (Ahern et al. 1985; Shirley, 1992). In this sense, it can be speculated that the irreversible alteration of protein structures influences protein digestibility and the final breakdown of peptides in free AAs.

TABLE 11 α AMINO GROUPS DETERMINATION IN SUPERNATANT AND PROTEIN DIGESTIBILITY (CALCULATED FROM THE DIFFERENCE BETWEEN TOTAL NITROGEN IN THE SAMPLE AND NITROGEN FOUND IN THE UNDIGESTED RESIDUE) ON EXTRUDED MATERIA

		μmol/ml		
	P dig. %	Tot. AA	Free AA	pept. AA
CTR	93.54	7.38	1.77	5.61
60	94.49	7.59	2.58	5.01
70	94.54	7.82	2.40	5.43
80	94.21	7.34	2.21	5.13
90	94.02	7.63	1.61	6.01

P DIG. – PROTEIN DIGESTIBILITY; AA – AMINO ACIDS

OM and DM digestibility (Table 16) were higher in extruded mixtures compared to the control (+ 16.8%). Wide differences between the control and treatments, and low CVs suggest a statistically significant increase of digestibility due to the extrusion treatment. The highest value for OM digestibility was recorded when extrusion was performed at 60°C (95.69%), increasing digestibility by 17.6 %. The expected increase in OM digestibility was likely due to the gelatinization of starch. As reported in literature (Lin et al., 1997), during extrusion processes starch gelatinization increases appreciably (30%) at temperatures close to 60°C.

TABLE 12 EFFECT OF BARREL TEMPERATURE IN EXTRUDED MIXTURES ON IN-VITRO DM/OM DIGESTIBILITY

	Rep	DM dig. %		OM dig	
		Mean	CV	Mean	CV
CTR	2	80.99	2.97	81.37	2.86
60	2	95.24	1.78	95.69	1.66
70	2	93.81	0.79	94.18	0.30
80	3	94.98	1.23	95.38	1.26
90	3	94.67	0.46	95.01	0.39

DM DIG. – DRY MATTER DIGESTIBILITY; OM – ORGANIC MATTER DIGESTIBILITY; CV COEFFICIENT OF VARIABILITY.

In light of the results, it can be concluded that 60°C is the best temperature for extrusion for the tested mixtures to remove the maximum amount of water and increase digestibility. Increasing the temperature beyond this threshold increases water retention without affecting digestibility, meaning that such increases are both useless and counterproductive in term of energy waste.

The low fat limit of 4.6 should be considered to obtain the most extrudable mixtures.

Implementation of the official methods for the detection of Processed Animal Proteins (PAPs) in feed

Experiment-1 Evaluation of microscopy in combination Image analysis for MBM characterization: comparison between bovine and swine material

The results obtained (Tables 13 and 14) indicated that out of 30 variables/descriptors measured on each lacuna, only 15 variables/descriptors were significantly ($P<0.001$) different between bovine and swine in terms of overall mean. Of these, 10 were primary descriptors including major axis, maximum diameter, maximum radius, perimeter, size length, perimeter 2, perimeter convex, perimeter ellipse, maximum feret, and mean feret. Five on the other hand were shape derived descriptors: aspect, area/box, radius ratio, form factor, and roundness. By contrast area, box X/Y, minor axis, minimum diameter, minimum radius, roundness, size width, perimeter ratio, area polygon, box width, box height, minimum feret, convex area and solidity did not differ between bovine and swine. These findings are very close to those observed in other studies on the same type of material from avian and mammalian by-products (Pinotti et al., 2007; Campagnoli et al., 2009; Van Raamsdonk et al., 2012; Pinotti et al. 2013). The results also indicated that 11 variables were bigger in bovine than in swine, except for area/box, form factor and roundness. Thus, values for all variables/descriptors measured in bovine were higher (+11% in terms of mean; $P<0.001$) than in swine. On the other hand, area/box, form factor and roundness were 11% smaller in bovine than swine. Our data thus indicate that not only are lacunae in bovine generally bigger than in swine but also that lacunae in this animal species differ slightly in shape. In fact several shape descriptors, such as aspect, roundness and form factor suggest that swine lacunae are more globular than in bovine.

TABLE 13 MEAN AND STANDARD DEVIATION (SD) BY SPECIES (BOVINE, BOV; SWINE, SUS) OF ALL LACUNAE PRIMARY DESCRIPTORS MEASURED.

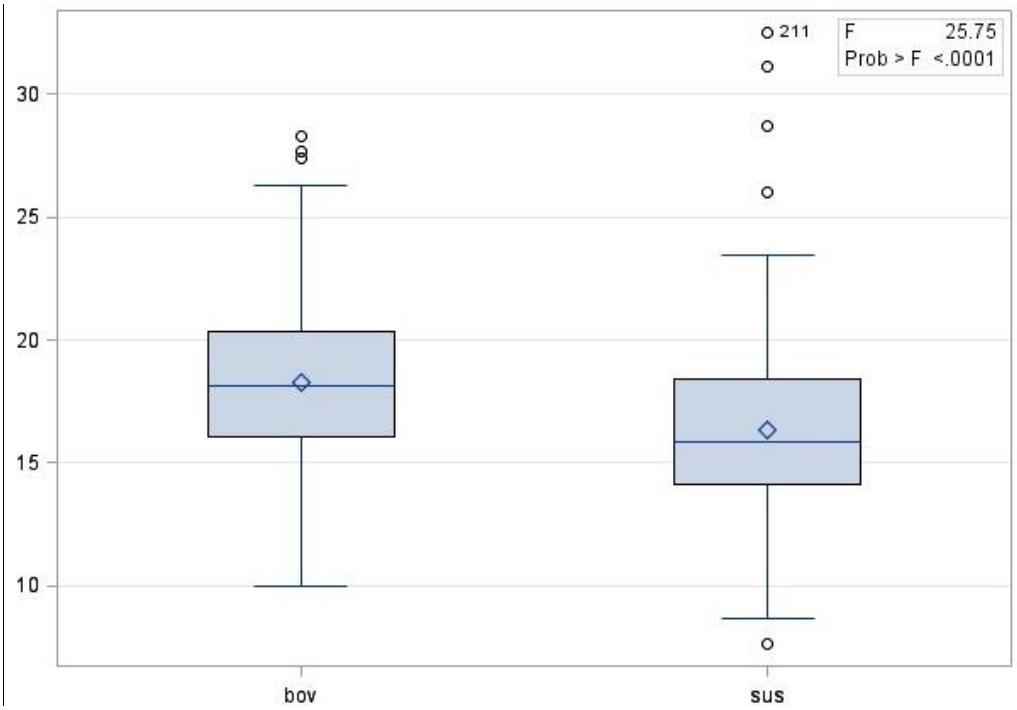
Variables/ descriptors	id	unit	BOV mean	BOV sd	SUS mean	SUS sd	P value
Area	V1	μm^2	102.15	33.64	93.85	27.77	0.0105
Axis major	V11	µm	18.24	3.30	16.35	3.71	< 0.0001
Axis minor	V12	µm	7.33	2.12	7.50	1.67	0.3826
Diameter max	V13	µm	18.85	3.54	16.74	3.89	< 0.0001
Diameter min	V14	µm	6.74	1.89	6.91	1.46	0.3396
Diameter mean	V15	µm	11.30	1.50	10.86	1.37	0.0043
Radius max	V16	µm	10.04	1.89	9.04	2.17	< 0.0001
Radius min	V17	µm	3.01	0.94	3.09	0.76	0.3364
Perimeter	V19	µm	49.91	10.82	45.14	11.15	< 0.0001
Size (length)	V28	µm	19.15	3.58	17.11	3.97	< 0.0001
Size (width)	V29	µm	8.30	2.39	8.23	1.90	0.7549
Perimeter 2	V30	µm	53.79	11.93	48.62	12.19	< 0.0001
Perimeter convex	V32	µm	44.53	7.10	40.91	7.79	< 0.0001
Perimeter ellipse	V33	µm	42.24	6.27	38.97	6.87	< 0.0001
Polygon area	V35	μm^2	96.28	32.75	88.48	26.88	0.0134
Box Width	V40	µm	14.65	4.80	13.78	4.61	0.0822
Box Height	V41	µm	14.18	4.83	12.68	4.23	0.0017
Feret (min)	V42	µm	8.17	2.33	8.10	1.80	0.7446
Feret (max)	V43	µm	19.20	3.56	17.18	3.95	< 0.0001
Feret (mean)	V44	µm	14.30	2.26	13.14	2.48	< 0.0001
Convex area	V57	μm^2	132.80	43.93	121.96	36.66	0.0109

TABLE 14 MEAN AND STANDARD DEVIATION (SD) BY SPECIES (BOVINE, BOV; SWINE, SUS) OF ALL LACUNAE DERIVED SHAPE DESCRIPTORS MEASURED

Variables/descriptors	id	BOV mean	BOV sd	SUS mean	SUS sd	P value
Aspect	V2	2.74	1.06	2.31	0.86	< 0.0001
Area/Box	V3	0.54	0.11	0.58	0.09	< 0.0001
Box X/Y	V4	1.26	0.80	1.28	0.77	0.7802
Radius Ratio	V20	3.76	1.68	3.18	1.46	0.0005
Roundness	V21	2.27	0.69	2.00	0.97	0.0033
Perimeter ratio	V34	0.88	0.07	0.90	0.06	0.0623
Form factor	V55	0.53	0.14	0.60	0.14	< 0.0001
Roundness 2	V56	0.41	0.15	0.48	0.15	< 0.0001
Solidity	V58	0.77	0.01	0.77	0.02	0.4597

However, probably 11% of differences are not detectable in routine lab practice, indicating that only differences can be detected with an image analysis approach/support. In the case studied in this paper, area and other primary descriptors that have been recently (Pinotti et al., 2013) proposed as key descriptors in distinguishing between animal classes (poultry and mammals), were not so effective, confirming that species identification needs an integrated approach (i.e. a combination of methods). Furthermore, in the studies in which mammalian and avian materials (distinguishing between class) have been tested (Pinotti et al., 2007; Campagnoli et al., 2009; Pinotti et al., 2013), the differences between variables were bigger than those measured between swine and bovine.

FIGURE 10 SELECTED PRIMARY DESCRIPTOR BOX PLOTS, NAMELY: LACUNAE AXIS MAJOR AND LACUNAE PERIMETER IN BOVINE (BOV), AND SWINE (SUS) SPECIES



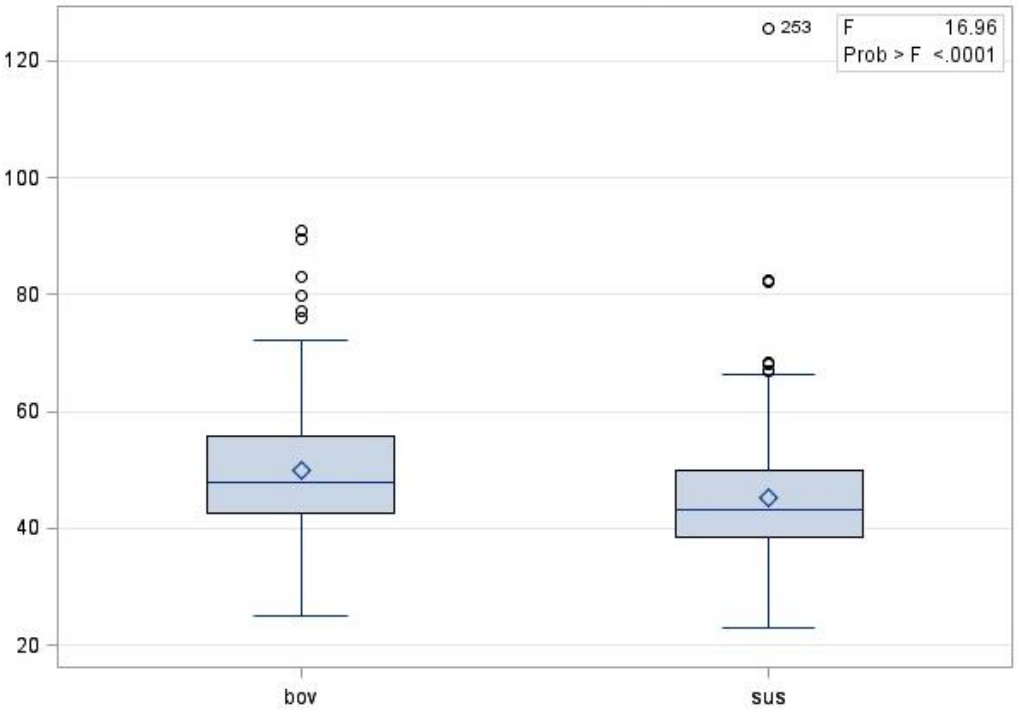
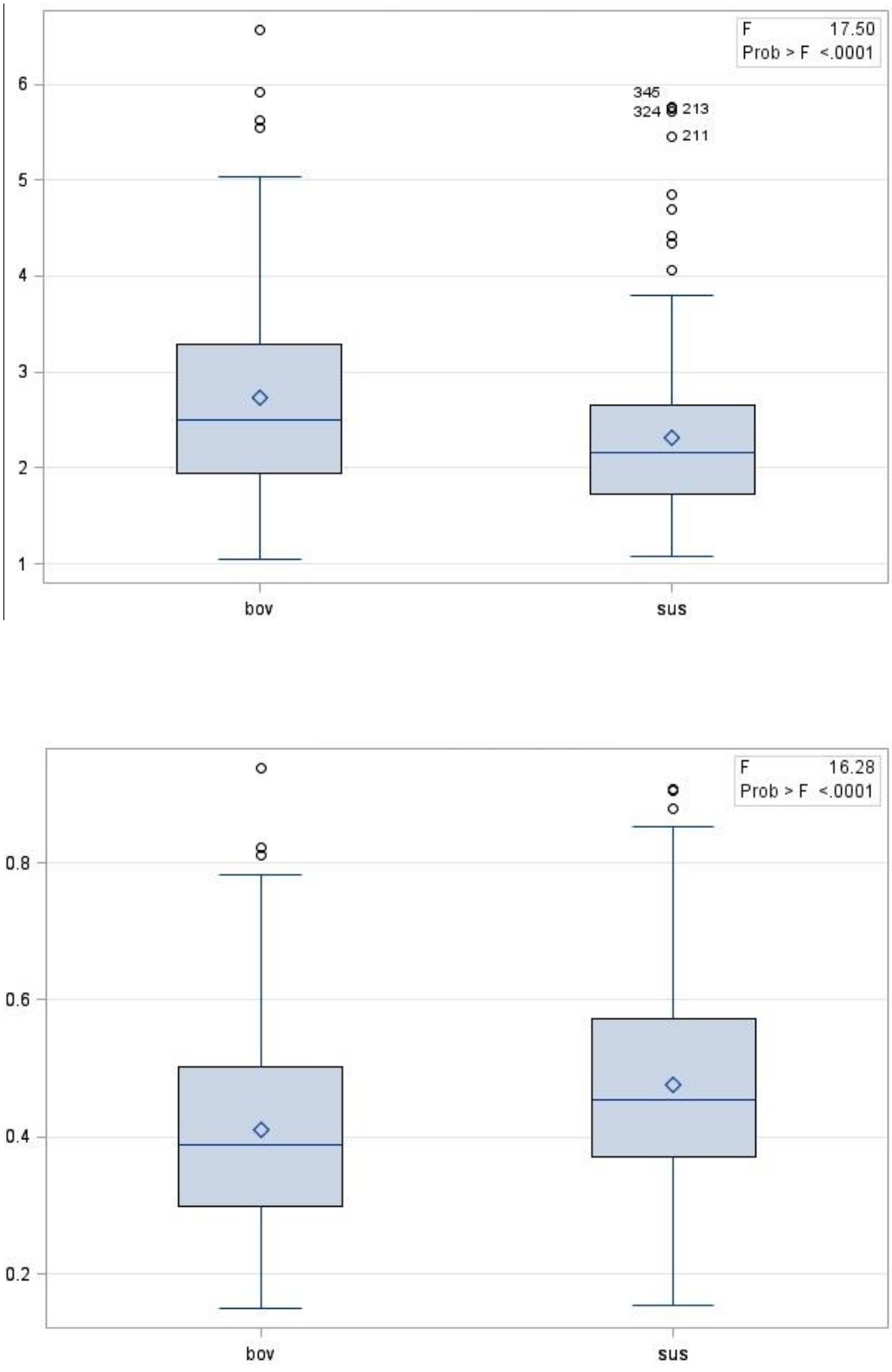


FIGURE 11 SELECTED DERIVED SHAPE DESCRIPTOR BOX PLOTS, NAMELY: LACUNAE ASPECT AND LACUNAE ROUNDNESS2 IN BOVINE (BOV), AND SWINE (SUS) SPECIES



This is supported by the more extensive investigation of the present dataset, which was performed using a box plot procedure. When for each variable mean, median, quartiles, and outliers were considered, as expected the dataset showed a considerable overlap

between species. In this respect, a few variables are presented in Figures 10 and 11. An analysis of these selected means, medians, and box plots clearly indicated that even though most of the variables measured were significantly different between bovine and swine in terms of overall mean, none of them per se is able to discriminate between species material (i.e. bovine vs swine). These results therefore confirm other findings (Pinotti et al., 2013) in the field, in which no clear indication of species differences within classes has been reported.

Experiment-2 Characterization fish material and comparison with land animal material

The results obtained by combining the microscopy method and image analysis for fish material characterization are reported in Tables 15 and 16. Of the 21 primary descriptors reported, 8 did not show any difference between samples. Specifically, diameter mean, radius max, radius min, perimeter (different types) and area polygon were comparable among the 6 samples. In the case of the other 13 descriptors of the same group, some significant ($P<0.001$) differences between samples were observed. When shape descriptors were considered, the situation did not change. All shape descriptors related to the radius and diameter were not significant, while Aspect, Area/Box, Box X/Y, perimeter ratio and roundness2 differed ($P<0.001$) among the samples. However, significant differences in observed values were not systematically distributed across different descriptors. Although there are some significant differences between means, the boxplots for all the variables showed very substantial overlaps between the distributions of measurements in the six samples. The boxplots for axis major and formfactor presented in figures 12 are typical examples.

TABLE 15 RESULTS SIZE DESCRIPTORS (PART A)

		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample6	
	unit	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Area	µm2	107.11a	4.51	85.79b	4.37	104.23	4.51	97.16	5.68	98.16	4.90	104.85	4.33
Axis Major	µm	33.48	1.17	32.34	1.13	30.11	1.17	28.85a	1.47	31.87	1.27	35.02b	1.12
Axis Minor	µm	4.64	0.18	3.86a	0.17	5.20b	0.18	4.77	0.23	4.57	0.20	4.59	0.17
Diameter Max	µm	36.31	1.34	35.61	1.30	34.68	1.34	31.55a	1.69	35.55	1.45	39.03b	1.28
Diameter Min	µm	4.13	0.17	3.59a	0.16	4.52b	0.17	4.26	0.21	4.01	0.18	3.93	0.16
Diameter Mean	µm	10.62	0.21	9.88	0.21	10.57	0.21	10.17	0.27	10.38	0.23	10.69	0.20
Radius Max	µm	20.02	0.75	19.61	0.72	19.17	0.75	17.77	0.94	19.90	0.81	21.66	0.72
Radius Min	µm	1.62	0.09	1.38	0.09	1.81	0.09	1.70	0.12	1.61	0.10	1.45	0.09
Perimeter	µm	85.76	3.61	81.74	3.50	87.90	3.61	79.23	4.55	87.94	3.92	93.83	3.47
Size Length	µm	37.08	1.33	36.74	1.29	35.46	1.33	32.31a	1.67	36.55	1.44	40.33b	1.27
Size Width	µm	6.32	0.34	5.21a	0.33	7.70b	0.34	6.88	0.43	6.55	0.37	6.75	0.33
Perimeter 2	µm	93.72	4.22	88.61	4.09	96.53	4.22	85.77	5.31	98.04	4.58	104.82	4.05
Perimeter Convex	µm	77.43	2.70	75.49	2.61	74.72	2.70	68.29	3.40	76.27	2.93	84.01	2.59
Perimeter Ellipse	µm	68.89	2.29	66.14	2.22	62.73	2.29	59.91	2.88	65.71	2.49	71.87	2.20
Area Polygon	µm	101.92	4.35	80.64	4.22	99.04	4.35	92.00	5.48	93.18	4.73	99.92	4.18
Box Width	µm	31.30ac	1.63	24.88	1.58	22.12bc	1.63	23.00	2.06	29.47c	1.77	21.49b	1.57
Box Height	µm	18.93a	1.69	25.16ab	1.64	24.80ab	1.69	20.54a	2.13	20.56a	1.84	31.39b	1.62
Feret Min	µm	6.35	0.32	5.24a	0.31	7.59b	0.32	6.73	0.41	6.51	0.35	6.72b	0.31
Feret Max	µm	37.10	1.33	36.75	1.28	35.47	1.33	32.35a	1.67	36.57	1.44	40.35b	1.27
Feret Mean	µm	24.72	0.86	24.09	0.83	23.87	0.86	21.80a	1.08	24.36	0.93	26.80b	0.82
Convex Area	µm2	155.16	7.33	123.84a	7.10	155.03	7.33	139.02	9.23	145.56	7.96	158.81b	7.03

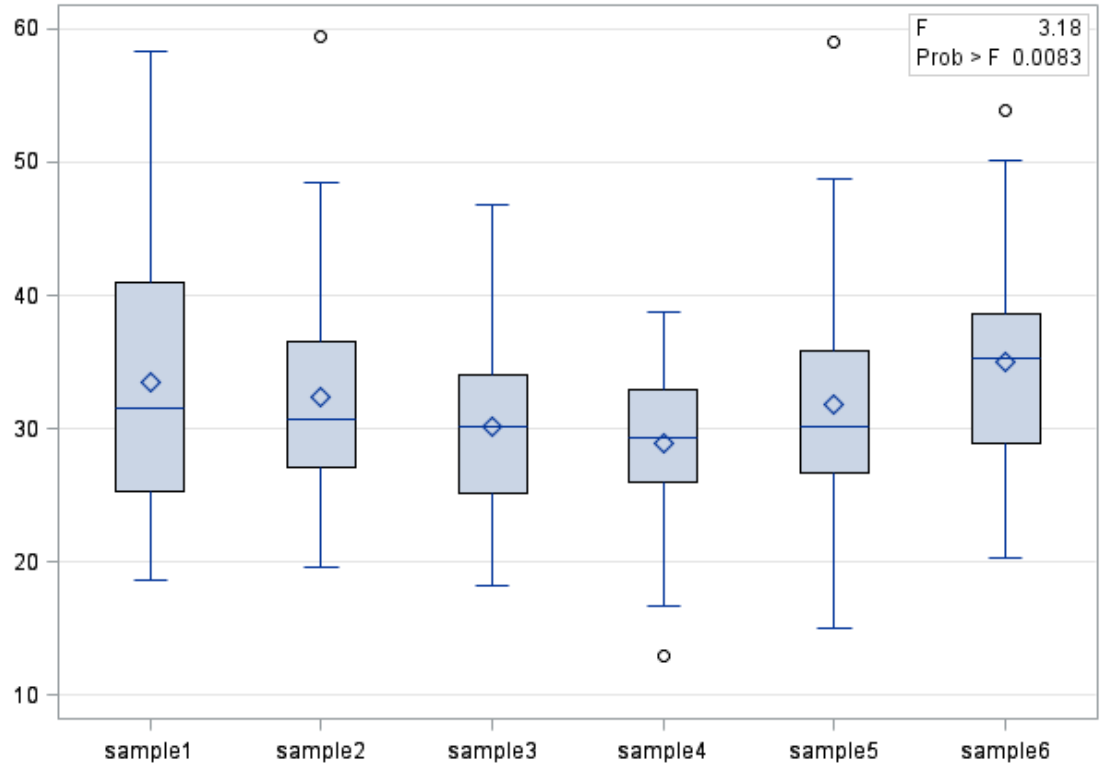
SEM, STANDARD ERROR OF MEANS; A,B,C, MEANS WITH DIFFERENT LETTERS DIFFER AT P<0.001

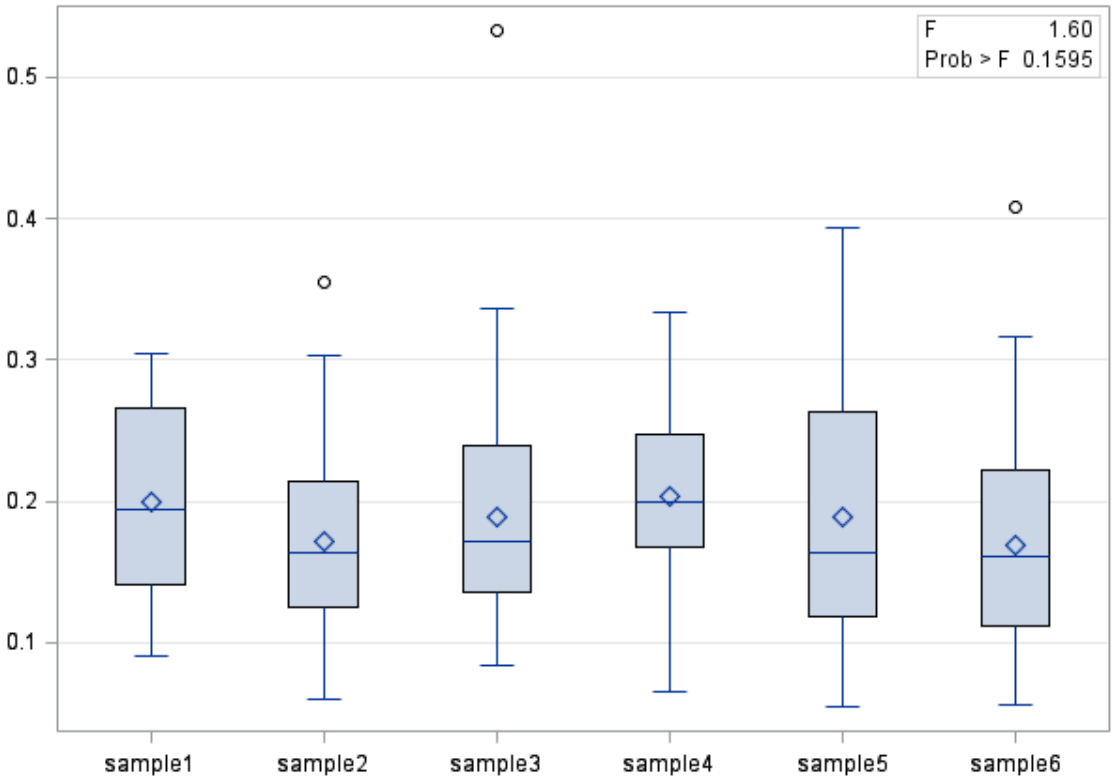
TABLE 16 RESULTS SHAPE DESCRIPTORS (PART A)

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Aspect	7.61	0.41	9.00a	0.40	6.18b	0.41	6.41b	0.51	7.46	0.44	8.11	0.39
Area/Box	0.23	0.02	0.18a	0.02	0.27b	0.02	0.28b	0.02	0.22	0.02	0.23	0.02
Box X/Y	2.13a	0.19	1.13b	0.19	1.38	0.19	1.42	0.25	1.63	0.22	1.05b	0.18
Radius Ratio	14.98	1.34	16.01	1.32	12.93	1.34	12.20	1.69	14.49	1.48	18.60	1.30
Roundness	6.08	0.45	7.28	0.43	6.59	0.45	5.99	0.56	7.21	0.49	7.60	0.43
Perimeter Ratio	0.90	0.01	0.92a	0.01	0.86b	0.01	0.87	0.01	0.88	0.01	0.90	0.01
Formfactor	0.20	0.01	0.17	0.01	0.19	0.00b	0.20	0.01	0.19	0.01	0.17b	0.01
Roundeness 2	0.13	0.01	0.11a	0.01	0.16b	0.01	0.15bc	0.01	0.14	0.01	0.12ac	0.01
Solidity	0.70	0.01	0.70	0.01	0.68	0.01	0.70	0.01	0.69	0.01	0.67	0.01

SEM, STANDARD ERROR OF MEANS; A,B,C, MEANS WITH DIFFERENT LETTERS DIFFER AT P<0.001

FIGURE 12 BOX-PLOTS DISPLAYING MEAN, MEDIAN, QUARTILES, MINIMUM AND MAXIMUM OBSERVATIONS AND OUTLIERS FOR AXIS MAJOR AND FORMFACTOR VALUES MEASURED IN SAMPLES 1 TO 6 IN PART A





Comparisons among fish, mammalian and poultry materials are presented in Figures 13, 4, and 15. For 17 of the 21 primary descriptors reported, there were significant differences in mean between fish and each of the terrestrial materials. Axis Minor and Diameter Mean did not differ significantly between fish and poultry materials and Area and Area Polygon did not differ significantly between fish and mammalian materials. Considering the shape descriptors, 8 of the 9 descriptors were significantly different in mean in the fish material compared with poultry and mammals; the only exception was the shape descriptor Box X/Y whose mean did not differ significantly from those of either of the other materials.

FIGURE 13 GRAPHIC REPRESENTATION OF MEANS AND STANDARD ERROR (SE) BY CLASS OF SOME (10 OF 21) OF THE SIZE DESCRIPTORS MEASURED IN PART B. AVI = AVIAN; FISH = FISH; MAM = MAMMALS; * = 10 TIMES THE MEASURED VALUE. THE MEANS WITHIN MORPHOMETRIC DESCRIPTORS WITH DIFFERENT LETTERS (A, B, C) DIFFER SIGNIFICANTLY (P < 0.001)

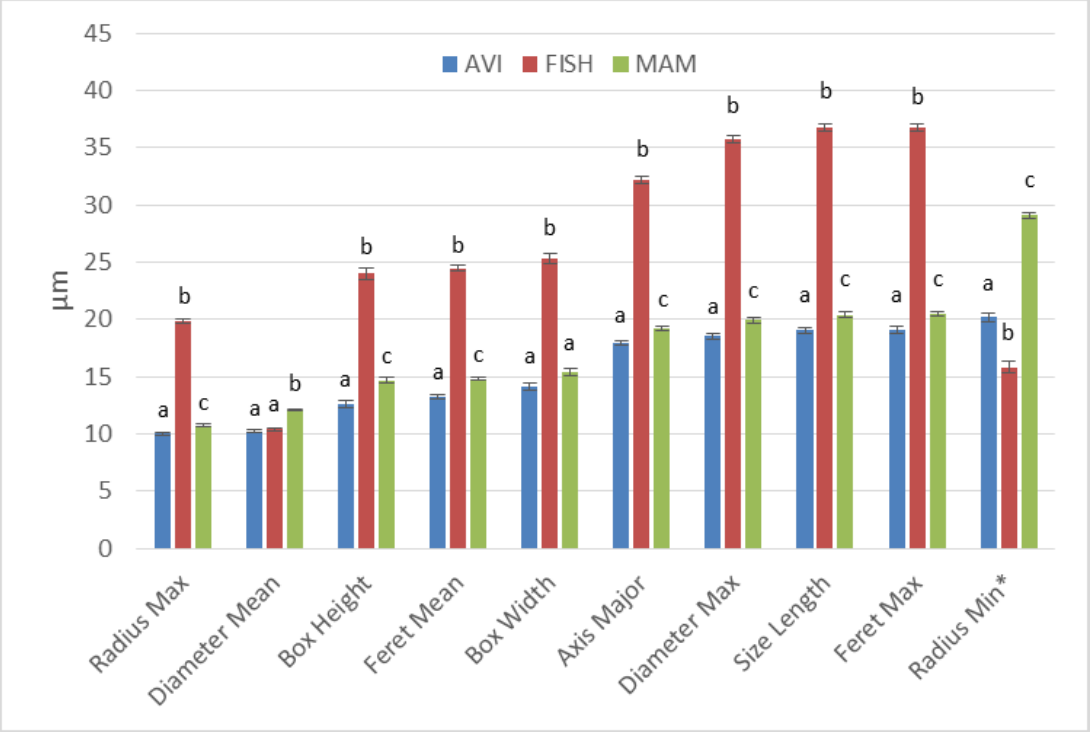


FIGURE 14 GRAPHIC REPRESENTATION OF MEANS AND STANDARD ERROR (SE) BY CLASS OF SOME (11 OF 21) OF THE SIZE DESCRIPTORS VALUE MEASURED IN PART B. AVI = AVIAN; FISH = FISH; MAM = MAMMALS; * = 10 TIMES THE MEASURED VALUE. THE MEANS WITHIN MORPHOMETRIC DESCRIPTORS WITH DIFFERENT LETTERS (A, B, C) DIFFER SIGNIFICANTLY (P < 0.001)

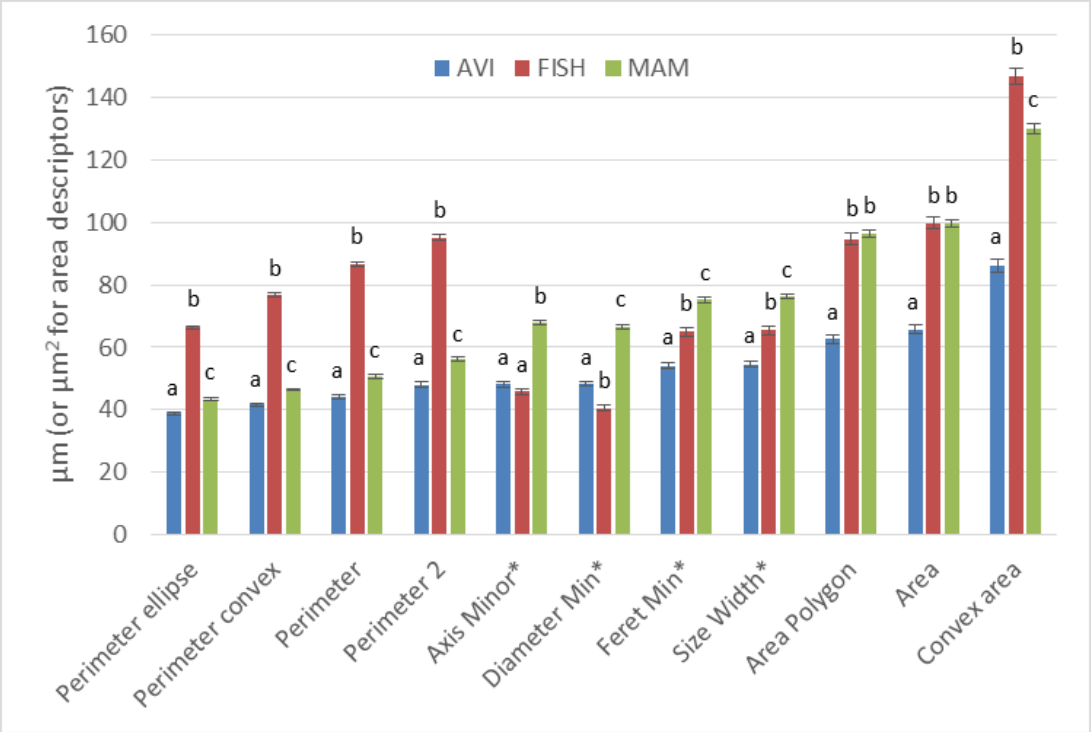
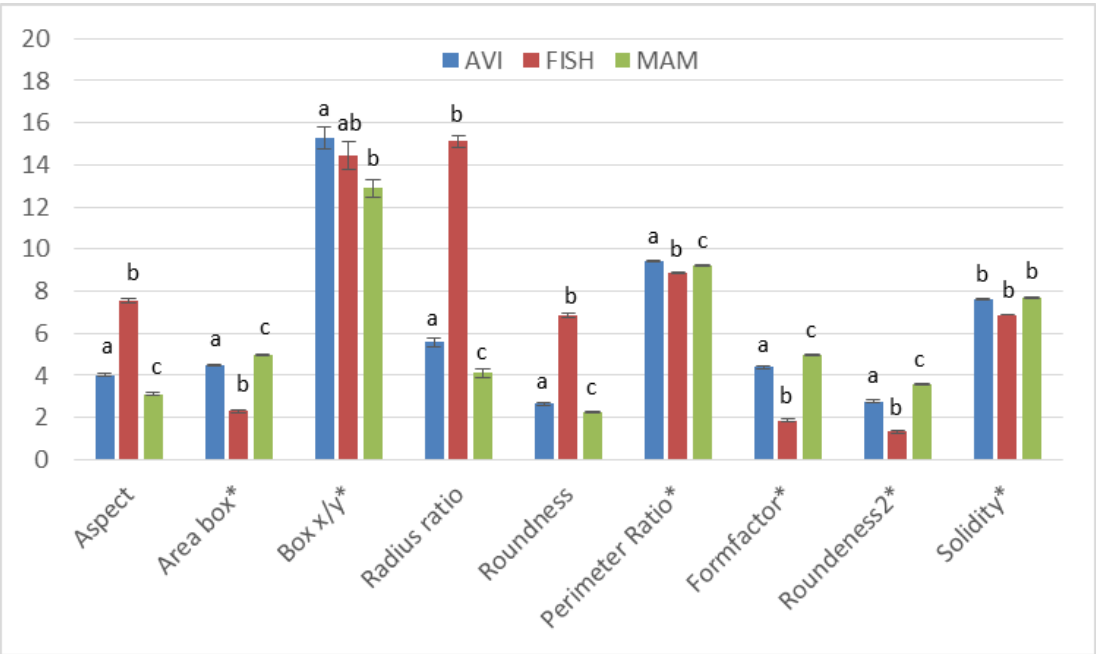
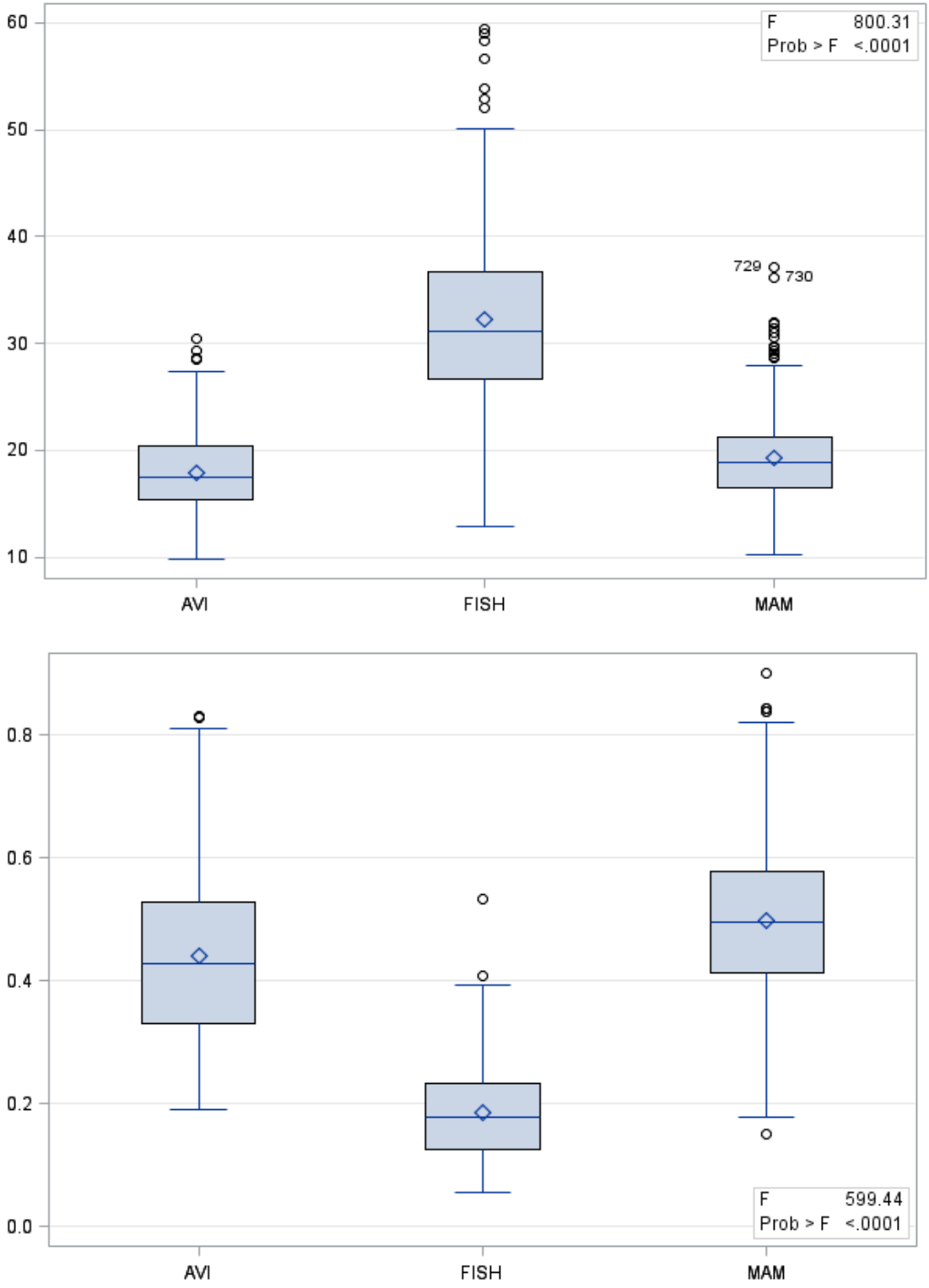


FIGURE 15 GRAPHIC REPRESENTATION OF MEANS AND STANDARD ERROR (SE) BY CLASS OF THE SHAPE DESCRIPTORS MEASURED IN PART B. AVI = AVIAN; FISH = FISH; MAM = MAMMALS; * = 10 TIMES THE MEASURED VALUE. THE MEANS WITHIN MORPHOMETRIC DESCRIPTORS WITH DIFFERENT LETTERS (A, B, C) DIFFER SIGNIFICANTLY (P < 0.001)



An analysis of the box plots showed overlap of the distributions for some descriptors but good separation for others. When fish material was compared to mammalian and poultry materials, a substantial overlap of the distributions of the measurements was observed for eleven morphometric descriptors. These were Area, Box X/Y, Axis Minor, Diameter Minor, Diameter Mean, Radius Minor, Size width, Area Polygon and Feret Minor Convex Area among the primary descriptors, and Perimeter ratio among the secondary ones. In contrast, very little overlap was observed for the other nineteen descriptors. In more detail, among primary descriptors, fish material showed very little overlap with land animal material for aspect, area box, axis major, diameter max, radius max, perimeter, size length, perimeter 2, perimeter convex and perimeter ellipse, Feret max and Feret mean. For secondary descriptors, all except perimeter ratio showed very little overlap between fish material and land animal materials. Specific examples are presented in Figures 16.

FIGURE 16 BOX PLOTS DISPLAYING MEAN, MEDIAN, QUARTILES, MINIMUM AND MAXIMUM OBSERVATIONS AND OUTLIERS FOR AXIS MAJOR AND FORMFACTOR VALUES MEASURED IN AVIAN, FISH AND MAMMALIAN SAMPLES IN PART B. AVI = AVIAN; FISH = FISH; MAM = MAMMALS

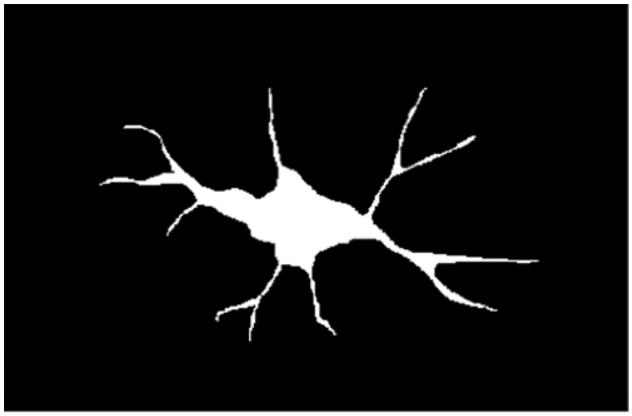


As expected, aquafeed composition was quite homogeneous among the 6 samples considered in the present experiment. Although 18 of the 30 descriptors measured on fish lacunae showed significant differences in mean between the 6 samples (Tables 13-14), the box plot analysis showed a substantial overlap between the 6 for the within-sample distributions of compound fish feeds analyzed in part A. These findings may be linked to the homogeneity of the fish meal used in aqua feed formulation (Ayadi et al., 2012). Indeed fish meal used in aquafeed production derives principally from pelagic fish and is then further treated to obtain a limited variety of standardized meals for the market. In addition, fish meal is produced by a few major players, using fish coming from three macro regions: Latin America (Peru and Chile), Scandinavia (Denmark and Norway) and Iceland (FAO Globefish, 2014). This scenario may contribute to standardization of fish meals used in compounded formulations, even though that is merely a speculative assumption. Aquaculture feeds are made using small pelagic forage fish, in part to produce farmed fish with levels of omega-3 long chain polyunsaturated fatty acids that are equivalent to those of their wild counterparts (Tacon and Metian, 2008). These forage fish come from a global supply that is expected to remain static or decrease over time (Tacon and Metian, 2008; Deutsch et al., 2007), while its features (in term of fish bone lacunae) seem to be quite stable.

A further result obtained in the present experiment is a detailed characterization of fish material in aquafeed. Observed values for some specific descriptors such as those related to axis, length and size, diameter, and Feret have indicated that fish bone lacunae in the analyzed samples were characterized by a very large length-width ratio. In the same way, Roundness 2, which is a derived shape descriptor calculated by combining area and axis major, provides a further indication about fish material features. The overall mean of the shape descriptor Roundness 2 in the fish samples was close to zero. Roundness 2 has a value of 1.00 for an ideal circle or a "circle like" shape. In contrast, smaller values indicate a greater departure from this ideal (Neal and Russ, 2012). Thus, the very low values observed in the present experiment in all the analyzed samples indicate elongated lacunae (high axis major - axis minor ratio), although a contribution from extended perimeters in fish lacunae cannot be excluded (see below). These findings imply a flattened and elongated to oblong shape for fish lacunae. This is in line with other authors (Gizzi et al., 2003; Jørgensen and Baeten, 2012) who have described fish bone lacunae as elongated with a clear fusiform net of canaliculae, although in several species lacunae are linear without visible canaliculae.

In this respect, IA seems to support the narrative shape description of fish bone lacunae in the literature. One more interesting shape descriptor for fish lacunae is represented by formfactor. The overall mean of formfactor in the fish samples is 0.18. Formfactor is a shape descriptor based on measurements of area and perimeter. Briefly, for shapes with identical areas the value of formfactor decreases as the apparent irregularity of the boundary and depth of indentations (and thus the length of the perimeter) increases. In this sense, fish bone lacunae seem to be star-shaped. Moreover, this numerical evidence is probably attributable to the presence of a fusiform net of canaliculae, confirming the narrative description reported by Gizzi et al. (2003). Therefore, combining these two shape descriptors, it can be concluded that the fish bone lacunae analyzed in the present experiment appeared elongated and/or star-shaped (figure 17).

FIGURE 17 EXAMPLES OF MONOCHROME MASK OBTAINED USING THE IMAGE ANALYSIS SOFTWARE (IMAGE-PRO PLUS 7.0; MEDIA CYBERNETICS INC., ROCKVILLE, MD, USA) FROM FISH BONE LACUNAE



With regard to part B, for the "length" descriptors (Axis major, Diameter max, Radius max, Size length, Box Height, Feret max), values from fish were twice as large as those from terrestrial animals, indicating that bone lacunae are significantly longer in fish than in terrestrial animals. Moreover, boxplot analysis indicated that the distributions of these size descriptors in fish had very little overlap with those from the terrestrial animals, suggesting these specific descriptors as valid markers for identifying and discriminating between fish and terrestrial particles. Moving to shape descriptors, for several of them (aspect, radius ratio, roundness, formfactor and roundness2) a large gap between fish and mammalian and avian material has been observed. For example, the shape descriptor Roundness 2 indicated that fish bone lacunae are more elongated than the lacunae of terrestrials (mammalian and poultry), which are oval to elliptical. Considering the descriptor formfactor, a large distance was observed between values

recorded in fish and terrestrials. This last evidence suggests that the shape descriptor formfactor could represent a valid marker for fish material identification.

Experiment-3 Characterization of insect material by microscopy and comparison with marine organism material.

Evaluation of an analytical approach for tracing insect material in feed.

Hermetia illucens larvae materials are shown in Figure 19, 21, 23 and 24. The structure observed in *Hermetia illucens* material was similar to the palisade cells of canola described by Makowsky et al. (2011). They are four or five sided with thick walls and a broad lumen. This lumen, as observed in surface view, is wider than the thick walls. The structure of the thick walls gives the cells a honeycomb-like appearance. The color of these cells ranges from yellow to burgundy, with the majority having an orange-red shade. Nevertheless, in insect material, cell-like structures are four or five sided and the color ranges from gray-cream to brown and dark. Bristles, generally long, narrow and yellow, have also been observed in *Hermetia illucens* material. Notably, bristles can present in different colors, probably depending on the amount of air trapped inside them. From the proximal part to the distal one outside to the inside, color moves through yellow shades, followed by a black and yellow line in the middle. Yellow silk—a rather small part of the interior—is completely black. As mentioned before, the black color indicates the presence of air bubbles trapped within the bristles. In light of this, it is important to note that HI bristles could be confused with the other vegetable structures. As described by Klein and Marquard (2005), trichomes of certain plants, such as wheat, are thickened, conical, straight or slightly curved. Thus, particular attention should be paid when insect material is included in mixtures with vegetables (i.e., compound feeds) to avoid errors in identification. As reported above, alizarin staining was also tested on insect (*Hermetia illucens*) material. As mentioned above alizarin red colors several calcium mineral forms. It is reported that this staining reacts principally with hydroxyapatite (contained in bone) but also with calcium phosphates (e.g., tricalcium phosphate) (EURL-AP, 2013). Accordingly, the hypothesis behind this experiment was that this insect species could be colored using a calcium specific stain. Indeed calcium content in *Hermetia illucens* larvae is higher than 9% on dry matter basis (Finke et al., 2013). Nevertheless, after staining *Hermetia illucens* material with the alizarin solution, no coloration was observed. This phenomenon probably occurred because the amount of calcium are present in HI larvae was too

small or possibly that the mineral form of calcium contained in this insect species does not react with alizarin red.

With regard to the chlorazol black stain test performed on *Hermetia illucens*, as expected, the insect material stained dark black. As described above, chlorazol black is a stain with a high affinity for chitin (Thomas et al., 2008), which is abundant in *Hermetia illucens* (Finke, 2009).

Insect material stained with aniline blue did react with UV light. Insect fragments showed a brilliant light-blue coloration. As indicated by several authors, aniline blue (triphenylmethane dye) specifically binds (1→3)-β-D-glucans. Nevertheless, no evidence in the literature reporting the presence of (1→3)-β-D-glucans in *Hermetia illucens* was found. On the other hand, Hershberger (1946 and 1948) proposed using aniline blue either alone or in combination with other staining (Safranine or Fuchsin basic) as a differential stain for insect tissues, but in their study, dilute staining solution was injected in into the live insects to color only specific organs, making them stand out against the general white background. Notably, in that same study, no UV light was used, and only internal organs were colored, which probably indicates an aniline blue stained compound different from chitin, which is present in the exoskeleton. In light of these findings, it can be concluded only that the aniline blue staining protocol tested here and observed with UV light can stain *Hermetia illucens* material.

Shrimp material is presented in Figure 18, 20 and 22. These fragments were characterized by the presence of more-or-less transparent particles of the chitinous shells. These particles showed very fine lines intersecting at random angles and extending across the whole particle. Occasionally, lines would connect across several other lines, forming triangles and other geometric shapes. In some areas, there may be cross-hatching. These findings are in line with the description of krill and shrimp meal reported by Marowski et al. (2011). In Figures 20 and 22, several circle-shaped holes can be observed. The chlorazol black and alizarin red stain tests colored the shrimp fragments dark black and reddish-pink, respectively. These results were also expected results because the shrimp exoskeleton is naturally rich in both chitin and calcium (Watkins et al., 1982; Sagheer et al., 2009).

By combining these results obtained on a small set of samples and observations, it can be suggested that chlorazol black stain can be efficiently used to mark *Hermetia illucens* material, with the proviso that bristle structure morphology (i.e., bristles) appear very similar to some vegetable structures and represent a limit to the accuracy

of these techniques in insect material identification in complex matrices such as compound feed. Alizarin red does stain shrimp fragments but did not stain the tested insect material, indicating a possible approach for discriminating between terrestrial and marine arthropods.

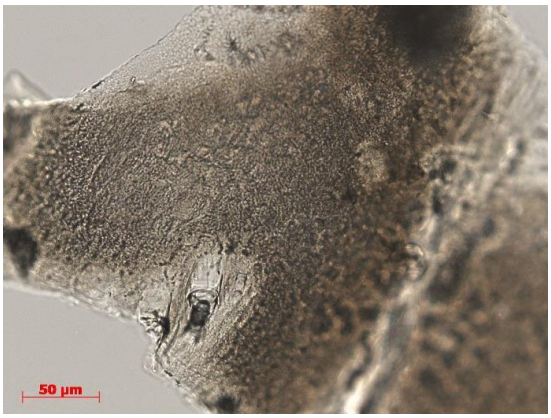


FIGURE 18 SHRIMP MEAL FRAGMENT, 40X MAGNIFICATION. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD

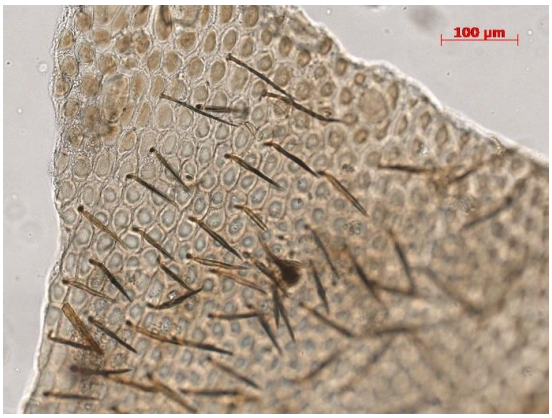


FIGURE 19 *HERMETIA ILLUCENS* FRAGMENT, 20X MAGNIFICATION. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD.

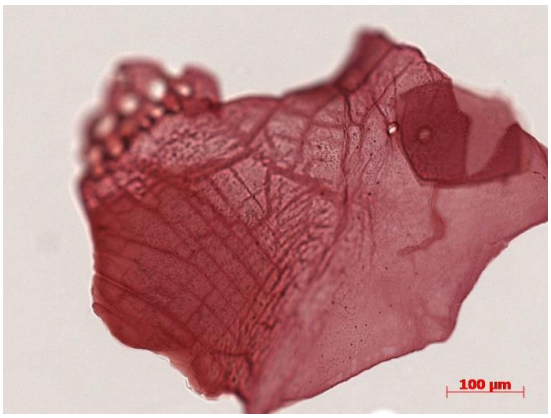


FIGURE 20 SHRIMP MEAL FRAGMENT, 20X MAGNIFICATION. ALIZARIN STAIN. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD.

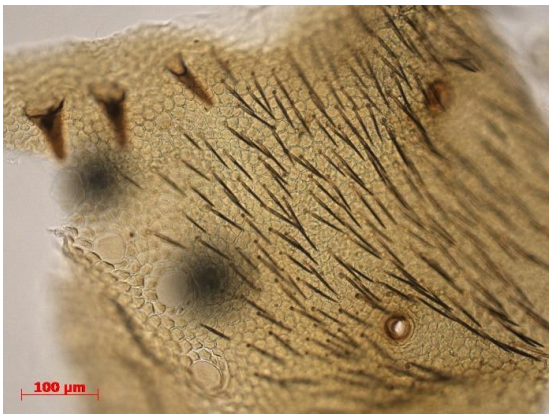


FIGURE 21 *HERMETIA ILLUCENS* FRAGMENT, 20X MAGNIFICATION. ALIZARIN STAIN. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD.

FIGURE 22 SHRIMP MEAL FRAGMENT, 20X MAGNIFICATION. CHLORAZOL BLACK STAIN. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD.

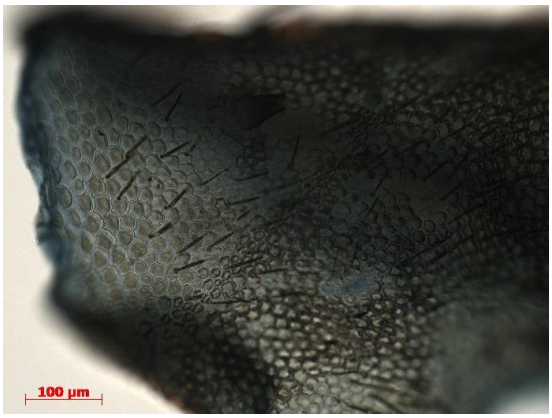


FIGURE 23 *HERMETIA ILLUCENS* FRAGMENT, 20X MAGNIFICATION. CHLORAZOL BLACK STAIN. EMBEDDING AGENT: NORLAND OPTICAL ADHESIVE 65. BRIGHT FIELD.

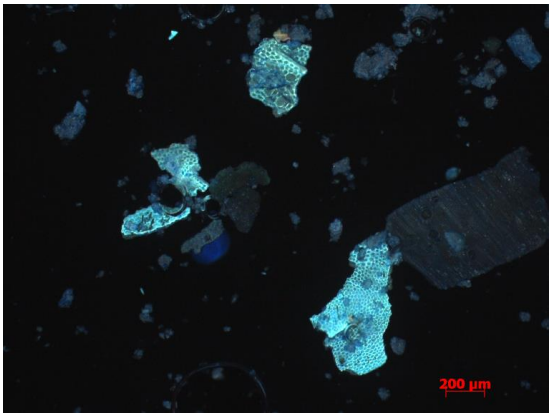


FIGURE 24 *HERMETIA ILLUCENS* FRAGMENT, 5X MAGNIFICATION. ALIZARINE BLUE SOLUTION. UV LIGHT.

Survey study

Investigation of potential areas for Innovation in Research and Development in the feed sectors

One hundred and thirteen feed companies were contacted. Of these, 37% responded by filling out the questionnaire, which generated 464 data points for Italian (IT) companies and 319 records for Serbian (RS) feed companies. The largest contribution was from small companies (45% of total responses) and the least from medium-size companies (40% of total responses). However, the response proportions are representative of the actual feed sectors in both countries.

The results are presented as CO, P&P and R&D. Regarding the CO, the results obtained indicated that Serbian firms were founded more recently than the Italian ones (mean ± SD, 1980 ± 30 and 2000 ± 14 years, for IT and RS, respectively). Serbian industries are all led by males (100%); in contrast, Italian industries are led by both genders (63% and 38% male and female, respectively). In both countries, feed production is based mainly on use of raw materials such as ground corn and soybean meal. Responding Italian industries were mainly focused on ruminant feed production, whereas those in Serbia produce feed for all species, which accounts for the large differences in pig and poultry feed production shown in Figures 25 and 26. There was a high rate of responses for the “other” category, which likely includes fish and pets.

FIGURE 25 THE PRODUCTION IS MAINLY BASE ON THE USE

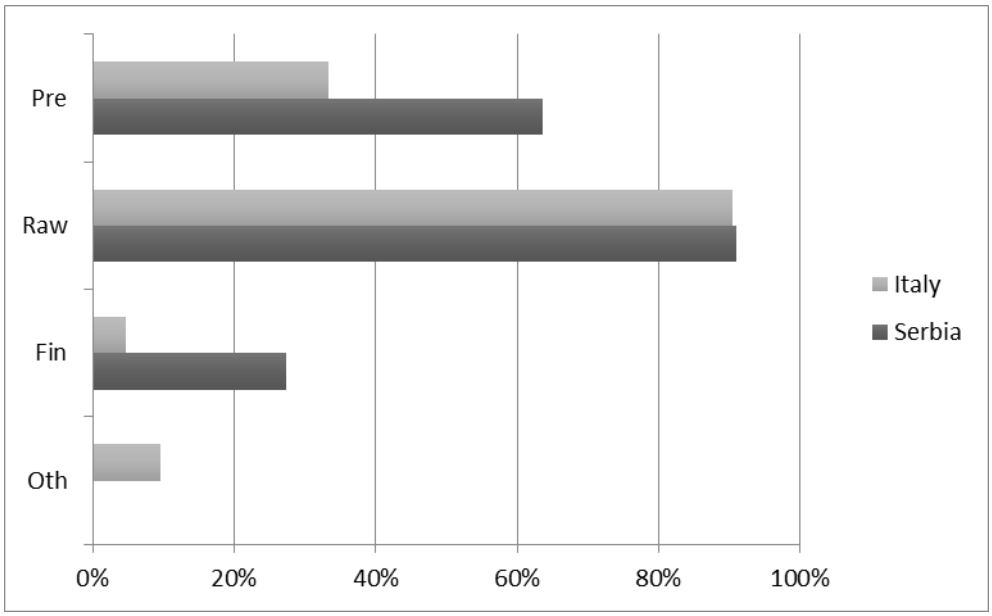
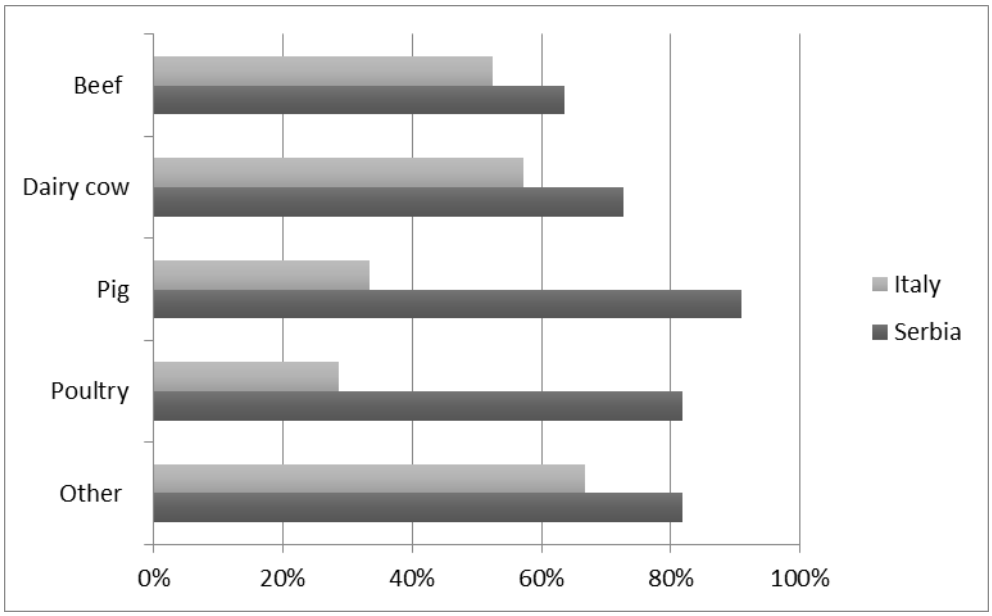


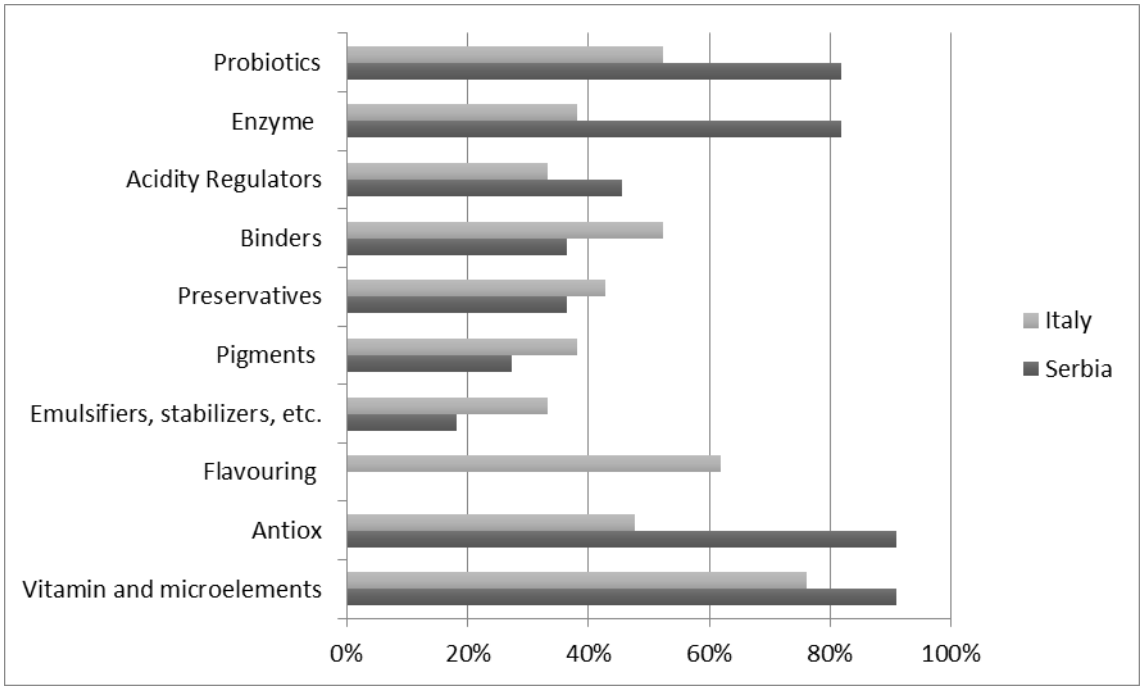
FIGURE 26 TYPE OF FEED PRODUCTION



In both countries, the use of feed additives is a common practice; 91% and 90% of responders (in Italy and Serbia, respectively) use feed additives in their formulations. When types of additives are considered, some differences for specific groups of additives were observed in both countries (Figure 27). The largest differences were observed for antioxidants, enzymes, probiotics and flavoring, although precise information within each class was not recorded.

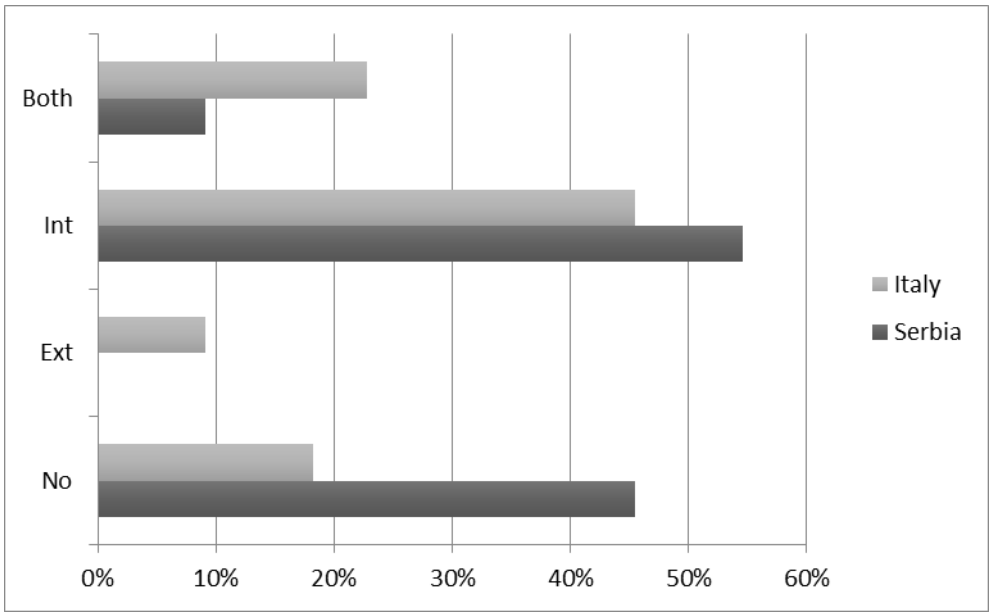
The differences in additives could be related to differences in the species for which compound feed is produced.

FIGURE 27 FEED ADDITIVES USED



Considering R&D in the past (Figure 28), 18% of Italian responders had not planned any budged for R&D, while for Serbian industries this percentage reached 45%. The main reason for this difference can be attributed to the fact that in both countries informal activities in R&D have been done in the past and their proportion could have been higher in Serbia than in Italy.

FIGURE 28 DISTRIBUTION OF BUDGET FUNDING SOURCES IN THE PAST 3 YEARS



When the R&D activities were tested, 64% of Italian responders chose industrial processes as one of their activities, while 82% of Serbian activities participate in new product development.

For both counties, marketing strategies are important, with the majority of companies reporting that product quality, market image, new markets, and the safety of those markets are a part of their innovation activities. The main differences between the Italian and Serbian industries are related to efficiency including security, profit and cost reduction (Figure 29). These differences could be due to the more recent establishment of the Serbian companies.

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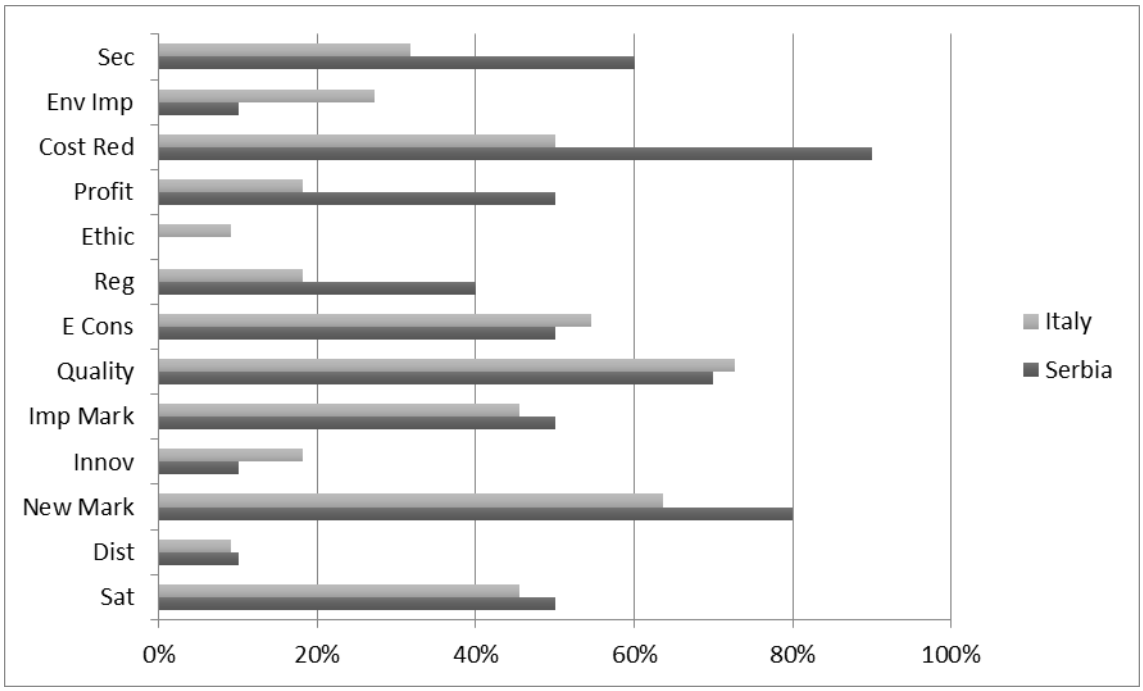
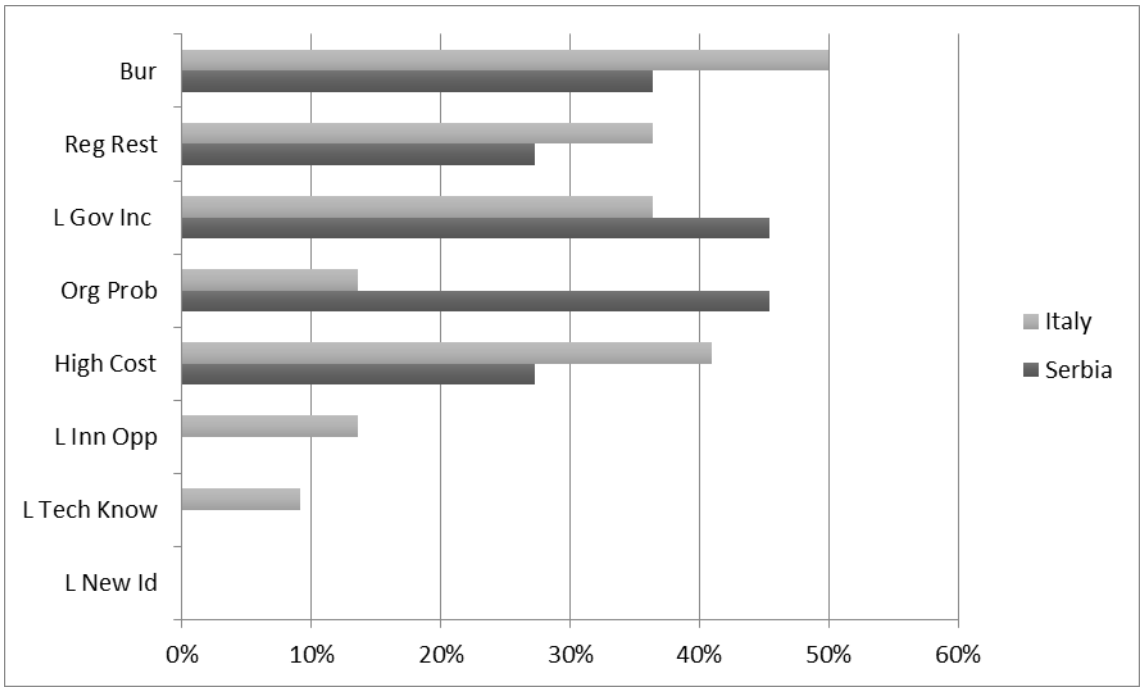
FIGURE 29 GOALS OF INNOVATION ACTIVITIES IN FUTURE



In general, these observations seem to reflect the different maturities and priorities of the feed market in the two tested areas. Approximately 20% of the feed industries consulted will not make any investment in R&D in the near future (next 3-5 years), but there will be unofficial R&D. This feature is common for both countries, in contrast with the differences in past investments. This discrepancy with the past is probably due to the need for Serbian companies to adapt their current production to new markets, such as the EU, which require new regulations, quality and safety standards. When type of feed production (monogastric, ruminants, etc.) was considered, research and development of new products in the dairy and beef cattle and pet sectors are the main areas in which Italian companies will invest in the next 3-5 years. The Serbian and Italian scenarios looks similar in regard to research and development of new products, extension/upgrade of production lines, new production technologies and control over production processes. The only exception is in advertising investment.

FIGURE 30 MAIN DIFFICULTIES ENCOUNTERED DURING INNOVATION ACTIVITIES



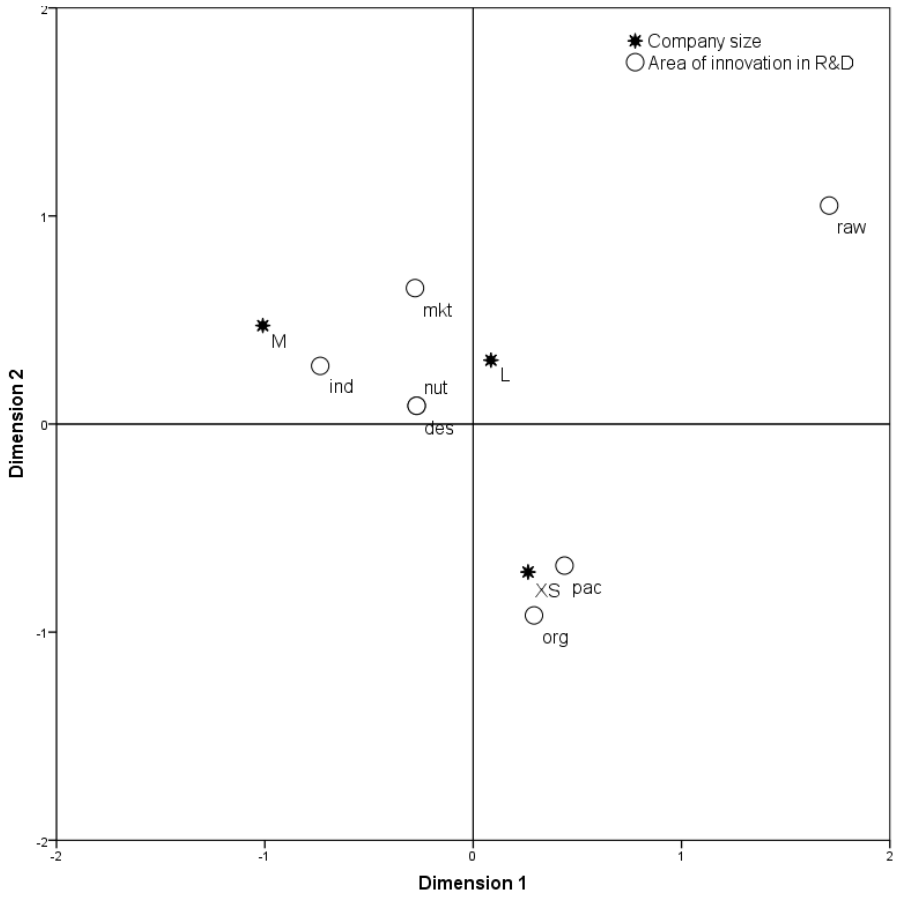
High costs of innovation, paperwork/bureaucracy, too-restrictive regulations and lack of government incentives were the main difficulties faced by industries in both countries (Figure 30). The main difference between the two countries is that organizational problems seem to be of more concern to Serbian companies.

The data for the correspondence between company size and areas of innovation in the last 3 years are shown in table 17; a graphical representation is presented in Figure 31. A significant correspondence ($P < 0.05$) was found among the considered categories, i.e., between company size and R&D target. The first two dimensions account for 87.5 % of the total inertia, using a considerably satisfactory quota of the raw information.

TABLE 17 STATISTICS OF THE BI-PLOT IN FIGURE 31

Category	Mass	Coordinate		Inertia	Contribution to dimension		Squared correlation		
		1	2		1	2	1	2	Quality
Company size									
XS	.361	.263	-.711	.080	.060	.501	.129	.829	.958
S	.012	4.108	2.877	.131	.489	.273	.646	.278	.925
M	.181	-1.010	.473	.103	.443	.111	.744	.143	.888
L	.446	.085	.307	.035	.008	.115	.038	.432	.470
Area of innovation in R&D									
raw	.084	1.709	1.050	.139	.592	.255	.735	.244	.978
des	.157	-.271	.088	.005	.028	.003	.889	.082	.970
ind	.181	-.734	.280	.060	.234	.039	.677	.086	.763
pac	.133	.438	-.681	.038	.061	.168	.278	.590	.868
mkt	.120	-.279	.653	.042	.023	.141	.093	.447	.540
nut	.157	-.271	.088	.005	.028	.003	.889	.082	.970
org	.169	.292	-.920	.060	.035	.391	.100	.870	.970

FIGURE 31 BIPLOT – CORRESPONDENCE BETWEEN COMPANY SIZE AND AREA OF INNOVATION IN R&D



The biplot in figure 31 shows a substantial differentiation among company size categories. In general, all categories, i.e., XS, S, and M, were clearly distinguishable from one another. This difference was exacerbated for the case of the S category, which was the smallest category considered in the study. By contrast, the L category was close to the origin, indicating a limited ability to distinguish one large company from another. By combining company dimension and areas of innovation, it was observed that the XS category was extremely close to the packaging (pac) and organization (org) points. This result indicates that XS firms have the most similar innovation in pac and company organization. In contrast, M is close to ind, indicating that medium firms are the most innovative in industrial processes. Large firms differ in the first two categories; indeed, as shown in the biplot, L is placed close to des and nut, indicating that they have innovated the most in product design (des) and nutritional content (nut) in the recent past.

The correspondence analysis figures (Table 17) show that XS and L reported a mass value of 0.36 and 0.45, respectively. As reported by Hoffman and Franke (1986), the mass is a weight of the number of times each variable was reciprocally connected by responders. Accordingly, we can conclude that XS and L are the most represented categories. On the other hand, the mass value of S was very low, probably due to the sample size. Considering the contribution to the dimensions of pac and org, in dimension 2, together they account for more than 0.42, indicating that packaging and company organization are both strategic in defining research and development efforts in XS firms. Notably, the *raw* component (research and development devoted to new ingredients) was characterized by high contribution to dimension and low mass values. This combination would suggest that even though raw material can be considered relevant in defining its position in the biplot (contribution to dimension), its mass is very low, which indicates that few companies in any size category innovated in raw material supply or uses in the past 3 years.

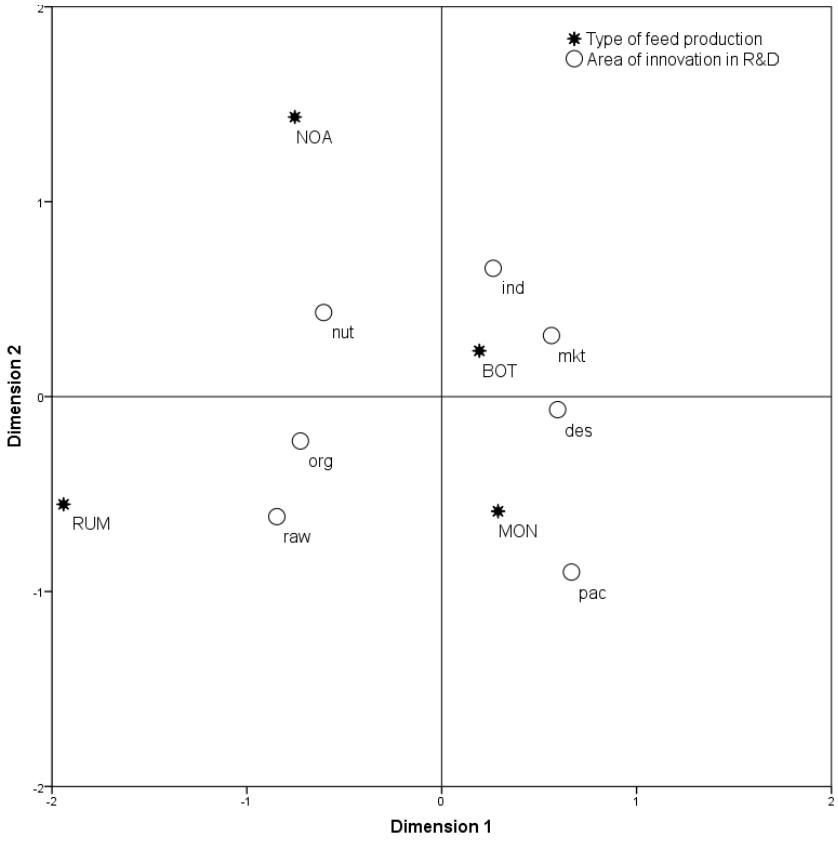
The data for the correspondence between type of production and the areas in which companies have performed the most innovation in the last 3 years are shown in Table 18 and graphically presented in Figure 32. No significant correspondence ($P > 0.05$) was found among the categories considered. Nevertheless, the first two dimensions account for 81.6 % of the total inertia. The map in Figure 32 does not show any substantial differentiation among company categories (type of feed production). Indeed, most of the points are condensed within a single area, indicating no

differentiation. Moreover, NOANSWER and RUM are isolated from the others indicating no correspondence with any points.

TABLE 18 STATISTICS OF THE BIPLOT IN FIGURE 32

Category	Mass	Coordinate		Inertia	Contribution to dimension		Squared correlation		
		1	2		1	2	1	2	Quality
Type of feed production									
MON	.292	.289	-.588	.050	.065	.371	.183	.553	.736
RUM	.079	-1.941	-.553	.119	.792	.088	.929	.055	.984
BOT	.573	.193	.235	.029	.057	.116	.277	.299	.576
NOA	.056	-.754	1.434	.064	.085	.425	.186	.489	.675
Area of innovation in R&D									
raw	.090	-.846	-.616	.040	.172	.125	.602	.232	.834
des	.146	.595	-.067	.023	.138	.002	.825	.008	.833
ind	.180	.264	.658	.031	.033	.286	.150	.679	.828
pac	.135	.666	-.900	.063	.160	.402	.354	.472	.826
mkt	.124	.563	.314	.035	.105	.045	.421	.095	.516
nut	.157	-.606	.432	.034	.154	.108	.635	.234	.869
org	.169	-.726	-.228	.036	.237	.032	.932	.067	.999

FIGURE 32 BIPLOT – CORRESPONDENCE BETWEEN TYPE OF FEED PRODUCTION AND AREA OF INNOVATION IN R&D



In general, combining the results and the answers obtained in the present survey suggests that, as expected, the main concerns in both countries are related to the economic balance between production costs and benefits. Indeed, the research and development needs manifested by the survey were focused on cost reduction, reducing energy consumption, improving quality, improving market image, development of new markets and satisfying market demand. Some small differences exist between the two countries—probably due to the greater maturity of the market in Italy versus its potential in Serbia. This seems to be confirmed by the Italian responders, for whom product quality is a further aspect that has been considered for R&D. However, matching the "innovation" needs manifested in the present study with literature and position papers from the feed sector and its associations (FEFAC, 2014; Connely, 2013) it is evident that some differences exist. Innovation through technological advances, development of novel ingredients, improved feed safety, increased automation and sustainability are the key factors not only according to feed associations (e.g., FEFAC) but also for multinational and large industries with predominant roles in the feed market (Connely, 2013). In the present study however, implementing automation, which has been proposed as a key innovation element for producing more feed and helping to ensure traceability, quality and biosecurity, was not mentioned by the companies involved in this survey. Aspects such as automation in combination with other technological advances in feed plants such as real-time automated verification systems, which have been considered as milestones for feed industries (Connelly, 2013), were not mentioned by either IT or RS feed companies. The same situation has been observed for the "new ingredients". Actually, for both Italian and Serbian feed companies the concept of resercah and innovation in new ingredients like insect, has not been mentioned. This is quite surprising since the general publich as well as the sector is sensibiklized to this themes are reported in different new papaers, magazines and on television. These discrepancies might be attributable to the sampled companies, which were primarily classified into the small and medium dimensions. In this segment of the feed sector, therefore, the research and development needs are more "basic" and are focused on the products and the main inputs (raw materials, energy etc.). Machinery and plant obsolescence are still relevant issues, but probably not over the short term, as respondents reported.

Conclusions

This PhD research project focused on the use of innovative technologies in feed production and formulation. Several aspects in the field of feed production were investigated: i) evaluation of new ingredients in feed formulation and the impact of technology on nutritional value, ii) improvements in the official methods for detecting processed animal proteins (PAPs), and iii) investigation of the innovation needs in research and development in the feed industry. Over the duration of my PhD program, I had the opportunity to address several different topics in collaboration with other institutions and laboratories, including Ghent University, the European Union Reference Laboratory for the detection of Animal Proteins in feedstuffs (EURL-AP), the National Reference Laboratory for Feed and Feed Additives (C.Re.A.A IZS Torino) and the Institute of Food Technology of Novi Sad, Serbia (FINS). By combining the results obtained in the various studies and trials, three main conclusions can be drawn.

Considering the technological aspects of including *Hermetia illucens* fresh material in an experimental extruded feed, the results vary. This study considered not only the technological aspects related to feed formulation and the extrusion process but also their impact on digestibility. In the extrusion experiment, increasing the blended fat content (up to 5.4%) reduced the NTV by four times (<100 Ncm) compared to 3.2 and 3.9% fat mixtures. The best performing mixture was larvae + wheat 25:75 (no added oil), containing 4.6% fat. When different barrel temperatures were considered for the same blend, no substantial effect on water loss was observed. Processing the mixture by extrusion increased OM digestibility by 13%, but not PD, compared to the untreated (no extrusion) mixture. Extrusion temperature did not affect either OM digestibility or PD. Extrusion can contribute to increasing OM digestibility in insect-containing feed blends. These results therefore indicate that that HI fresh material can be efficiently included in experimental extruded feed. Observation suggested that specific attention should be paid to the lipid content. Indeed, when the lipid content is lower than 4.6%, mixtures are not extrudable. Fat content in the mixture is a key variable that should be defined for the extrusion process. However, the present results were obtained at the lab scale and need to be confirmed in a large-scale pilot plant.

Moving to the second topic trial, as mentioned above, starting on 1 June 2013 (European Commission, 2013b), processed animal proteins (PAPs) from non-ruminants have been re-authorized for use as feed or feed ingredients in aquaculture. This is clearly a first step toward the re-introduction of non-ruminant PAPs not only in aquafeed but also in feed for poultry and pigs (IFFO, 2013), which could also enable the EU to decrease its dependence on other sources of protein (European Commission, 2010). To open the way to a possible easing of the regulations a consistent technique for identifying and quantifying levels of PAP in compound feeds that could be used by the member states analytical laboratories must be developed. The results obtained from microscopy trials could help drive the re-introduction of non-ruminant PAPs in feed. Accordingly, combining the official method (microscopy) with image analysis to detect constituents of animal origin in feedstuffs was investigated as an innovative tool for discriminating between bovine and swine lacunae. In light of the results obtained here, we conclude that microscopy methods—even if improved—are not able to meet all the requirements for the accurate identification of prohibited ingredients of animal origin, and therefore, a combinatory approach is recommended. In the same field, another experiment aimed at investigating the use of microscopy in combination with image analysis measurements for the characterization of fish bone lacunae in aquafeed-extracted material was performed. In this case, the results showed that using a combination of light microscopy and image analysis, fish material in aquafeed appears quite homogenous in term of bone features. Moreover, by selecting specific markers, fish material can be efficiently distinguished from avian and mammalian materials. Still, a larger dataset is needed for an exhaustive evaluation. The same analytical approach was evaluated for the characterization of insect materials (i.e., pure insect meal). The first basic assumption was adopted for this study was the "absence of bone lacunae" in insect material. Starting from this assumption, this study's results indicated that specific stains can be used to efficiently mark insect fragments (*Hermetia illucens)* material. Specifically, the present work tested alizarin, chlorazol black and aniline blue. In general, the results indicate that any of these specific stains can be recommended as gold standards, even in combination. Furthermore, the present work has also shown that mis-classification between shirmp and insect materials (from *Hermetia illucens)* can occur—but again, using a specific stain (alizarin) can help in distinguishing the two materials. In contrast, some similarity with vegetable structures was observed, representing a limit in insect material identification in complex matrices

such as compound feed. However, the same problem of similarity has also been encountered for other animal materials in feedstuffs. Notably, this experiment was a preliminary investigation based on a limited number of pure samples, thus further investigation based on a complex matrix (compound feed, and eventually, extruded feed) is required.

The last study carried out here concerned research and development efforts in the feed sector and their implications for innovation (innovation needs). To address these issues a targeted survey was performed in two countries: Italy and Serbia. Although these two countries are not fully representative of the entire European feed sector, Italy is the sixth largest compound feed producer in the EU (USDA, 2015), and Serbian feed production is among the largest in the Balkan area (Djuragic, 2014). Matching the "innovation" needs as manifested in the present study with literature and position papers from the feed sector and its associations (FEFAC, 2014, Connely, 2013), it is evident that some differences exist. Innovation through technological advances, new ingredients, improvements in feed safety, increased automation and sustainability are the key factors not only according to feed associations (e.g., FEFAC) but also for large multinational industries with a predominant role in the feed market (Connely, 2013). In the present study, however, the search for new ingredients such as insect material, which has been proposed as a key element for the near future in Europe (Insects to Feed the World Conference, 2014; FAO 2013; PROteINSECT, 2015), was not mentioned by the companies involved in the survey. This may be attributable to the predominantly small and medium sizes of the surveyed companies. In this segment of the feed sector, research and development needs are more "basic" and are focused on the products and the main inputs (classical raw materials, energy cost etc.). These results were also confirmed using simple correspondence analysis as an alternative/innovative tool for questionnaire analysis. As expected, the results show that innovation focus is related to a company's size. This aspect is particularly evident for extra-small companies. However, no correspondence was observed between type of production and area of innovation.

Based on the present research work, and in line with some position papers on this matter (Insects to Feed the World Conference, 2014) a wide range of socio-economic opportunities based on using insects are accessible at every scale of production in both developed and developing countries. These include creation of jobs, enterprise

development, food and animal feed production, organic waste processing and increased global trade. In spite of the advantages, several major challenges need to be addressed as well, including the legal status, raising awareness among the general public, ensuring the safety and quality of insect material for both food and feed, improved methods of analysis for characterizing insect meals, identification and traceability.

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List of publications

Published

Ottoboni M., Cheli F., Amato G., Marchis, D., Brusa B., Abete M. C., Pinotti, L. 2014. Microscopy and image analysis based approaches for the species-specific identification of bovine and swine bone containing material. Italian Journal of Animal Science, 13(3187): 377-381

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Submitted paper

Pinotti L., Ottoboni M., Caprarulo V., Giromini C., Gottardo D., Cheli F., Fearn T., Baldi A. 2015. Microscopy in combination with image analysis for characterization of fishmeal material in aquafeed. Submitted in Animal Feed Science and Technology ISSN: 0377-8401. Manuscript number: ANIFEE-15-6603.

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