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PORTUGAL

ANIMAL SCIENCE DOCTORAL PROGRAMME

- I WORKSHOP

16th September 2014

Organizers

António Mira Fonseca, REQUIMTE & ICBAS
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Conference Chairman

António Manuel de Sousa Pereira, ICBAS
Ana Rita Cabrita, REQUIMTE & ICBAS
Jorge Dias, Sparos

AGENDA

14:00 – Opening Session: **Chairperson António Manuel de Sousa Pereira**, ICBAS-UP Director
Baltazar Manuel Romão de Castro, REQUIMTE President
Vítor Manuel Oliveira Vasconcelos, CIIMAR Director

14:30 – Presentation of the new Doctoral ANIMAL SCIENCE Programme in an industrial setting funded by FCT: SANFEED - Sustainable Animal Nutrition and Feeding
António Mira Fonseca, REQUIMTE & ICBAS-UP and Luísa MP Valente, CIIMAR & ICBAS-UP

Session I: Chairperson Ana Rita Cabrita, REQUIMTE & ICBAS

15:00 – Plenary session: European Animal Feed Market – New trends and perspectives
Emídio Gomes, Portugal North Autorithy & ICBAS-UP

15:30 – Mediterranean Grain Legumes: Chemical Composition and Bioactive Compounds
Sara C. Q. Magalhães, REQUIMTE & ICBAS-UP PhD student

15:45 – Modelling the temperature and pH decline early post-mortem of the beef carcasses
Cristina Xavier, CIMO & ICBAS-UP PhD student

16:00 – Effects of salinity challenge on ion regulation in estuarine migrating sea lamprey, Petromyzon marinus
Diogo Martins, CIIMAR & ICBAS-UP PhD student

16:15 – Young of the Year (YOY) flounder condition and relationship with habitat features in the Lima estuary (NW Portugal)
Cláudia Mendes, ICBAS-UP PhD student

16:30 – Coffee Break

Session II: Chairperson Jorge Dias, *Sparos*

17:00 – Plenary session: Future directions in Fish Nutrition
Sadasivam Kaushik, INRA

17:30 - Use of probiotics intercropped with plant protein diets and their influence on the growth performance and immunological status of Senegalese sole, Solea senegalensis
Sónia Batista, CIIMAR & ICBAS-UP PhD student

17:45 – Development and establishment of an effective and reproducible infection model of Tenacibaculum maritimum against Senegalese sole (Solea senegalensis)
Mahmoud Mabrok CIMAR & ICBAS-UP PhD student

18:00 - Effect of seaweed supplementation on growth performance, immune and oxidative stress responses in gilthead seabream (Sparus aurata)
Augusto C. Queiroz, ICBAS-UP PhD student

18:15 - Protein hydrolysates affect protein accretion and the regulation of muscle growth in Senegalese sole larvae
Paula Canada, CIIMAR/CCMAR & ICBAS-UP PhD student

18:30 – Discussion: Chairperson Luísa MP Valente, *CIIMAR & ICBAS-UP*

ORAL COMMUNICATIONS

THE COMPOUND FEED INDUSTRY IN THE EU LIVESTOCK ECONOMY

Emídio Gomes

ICBAS-CCDRN

The compound feed industry has become capital intensive in recent years and makes use of a very high level of technology. Advanced methods are used to formulate feeds according to the demands of the livestock farmer – which reflects final consumers' demand – and to control the raw materials used, the manufacturing process and the quality of the finished feeds. The compound feed industry is subject to a complex body of both EU and national legislation affecting almost every part of its operation. This legislation is designed to ensure that feeds are of high quality and are safe for both livestock and consumers.
Within the EU-27, about 470 mio. t of feedingstuffs are consumed by livestock each year. Out of this quantity, 230 mio. t mostly are roughages grown and used on the farm of origin. The balance, i.e. 240 mio. t of feed, includes cereals grown and used on the farm of origin (53 mio. t) and feed purchased by livestock producers to supplement their own feed resources (either feed materials or compound feed).

In 2012, 153.5 mio. t of compound feed were produced by EU compounders, accounting for 82% of all purchased feedingstuffs. Purchases of compound feed amounted, in 2011, to €51 billion. Compound feed are manufactured from a mixture of raw materials designed to achieve pre-determined performance objectives among animals. These raw materials are obtained from a wide variety of sources. Hence, the industry provides a major market for EU cereals, oilseeds and pulses. Some raw materials are obtained from the co-products of the food industry. Other important ingredients which cannot be grown in sufficient quantity in the EU are imported from third countries. These diverse sources of raw material supplies are an important factor in the industry's ability to manufacture feeds of both high quality and at competitive prices for livestock farmers.

MEDITERRANEAN GRAIN LEGUMES: CHEMICAL COMPOSITION AND BIOACTIVE COMPOUNDS

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Introduction

Grain legumes (GL) are crops of the botanical family Fabaceae grown for food and feed. Their recognized high protein content is the reason why they are dubbed "poor man's meat" in low-income groups in developing countries (Tharanathan and Mahadevamma, 2003). In animal nutrition, GL also constitute appealing economical and sustainable alternatives to the protein sources commonly used (for instance soybean meal; e.g. Jezierny *et al.*, 2010). Mediterranean countries present suitable edaphoclimatic conditions for GL growth and measures towards increasing GL production have already been purposed in the European Union. Beyond proteins, legume seeds are great sources of energy and fiber (Jezierny *et al.*, 2010) also containing non-nutrient bioactive compounds that may exert positive, negative or both effects in those who ingest them (Champ, 2002). For instance, while carotenoids and phenolic compounds confer antioxidant properties to GL, other metabolites such as oxalates, enzyme inhibitors or alkaloids may decrease nutrients' availability and digestibility in the gastrointestinal tract. Therefore, it seems imperative to determine in detail the nutritive value of GL available to better include these ingredients in humans' and animals' diets. The aim of this work was to characterize several Mediterranean GL varieties regarding nutritional and bioactive properties. Whenever possible, Portuguese (PT) varieties were compared with those from foreign (F) countries.

Species chosen and analysis

Grain legumes studied included Kabuli (beige; n=5) and Desi (dark; n=1) chickpea (*Cicer arietinum*), field pea (*Pisum sativum*; n=21), faba bean (*Vicia faba* var. minor; n=10), white (n=5), narrow-leafed (n=2) and yellow (n=5) lupins (*Lupinus albus, L. angustifolius* and *L. luteus*, respectively), chickling vetch (*Lathyrus cicero*; n=1) and common vetch (*Vicia sativa*; n=1) which were provided by several companies from Portugal, Spain, France, Italy and Poland. Fourteen varieties belonged to the Portuguese Catalog of Varieties. Proximate composition was determined in all varieties as described by Cabrita *et al.* (2011). Seeds were also analyzed for fatty acids profile by GC, according to Alves *et al.* (2008), organic acids by HPLC-UV following Sousa *et al.* (2009), carotenoids by HPLC-DAD as described by Mariutti *et al.* (2012) and phenolic compounds by HPLC-DAD according to Silva *et al.* (2005).

Major results

Among all samples, protein content ranged between 21.0 and 42.8% DM with values above 32% belonging to lupins. Protein fraction was characterized as being highly soluble (62.7±5.41%) in all samples. Fattest samples were chickpeas and lupins (4.7±0.83 and 6.2±1.74% ether extract in DM, respectively) and the major fatty acids found in all varieties were palmitic (16:0), oleic (18:1c9) and linoleic (18:2) acids that accounted for more than 75% of total fatty acids. Within chickpeas, the dark variety, which is suited for animal feeding, presented less fat and starch contents and higher levels of cell-wall components than beige seeds. Starch content ranged from 27.3 to 44.6% DM in all samples, lupins being an exception. Indeed, lupins lacked starch but

presented increased amount of non-starch polysaccharides (17-29% DM) comparatively to the other samples. Major differences between PT and F varieties were observed in beige chickpeas; PT seeds presented (DM basis), in average, less 4.5 percent points (pp) of protein and more 1.4 pp of fat and 5.4 pp of starch, relatively to F ones. Also, PT faba beans and white lupins had lower protein content, while field peas and white lupins showed similar values between both groups.

Among all varieties, only two carotenoids were identified, namely lutein and zeaxanthin. Lutein was present in all samples and zeaxanthin only in chickpea and lupins. Of all chickpeas, dark variety stood out from the beige ones in terms of total carotenoids content (162.3 vs. 28.7-87.6 µg g^{-1} DM, respectively). Indeed, dark chickpeas present higher antioxidant activity (Segev *et al.*, 2010), carotenoids contributing for that. Main differences between PT and F varieties were also found in beige chickpeas, with the formers presenting higher total carotenoids levels.

Several organic acids were identified in GL seeds (Figure 1). Citric and aconitic acids (antioxidant agents) were common to all varieties, the former being the major compound in all samples. Lupins presented the highest total amount of organic acids (4.0±0.43 mg g^{-1} DM) and common vetch the lowest (0.5 mg g^{-1} DM). Among all, oxalic acid should be highlighted once it affects calcium and magnesium metabolism and protein digestion when ingested mainly by monogastrics (Akande *et al.*, 2010). Results showed field peas and faba beans to lack oxalic acid and the other species to contain between 2.0 and 7.7 mg 100 g^{-1}. These values are considered low for human consumption (OHF, 2008) and are below those found for soybean seeds (Massey *et al.*, 2001). PT yellow lupin presented higher organic acids content than F ones mainly due to increased citric acid levels.

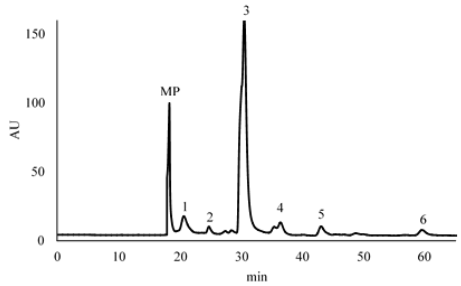

Figure 1. HPLC-UV organic acids profile of chickpea var. Elvar. Peaks identification: mobile phase (MP), oxalic acid (1), *cis*-aconitic acid (2), citric acid (3), malic acid (4), *trans*-aconitic acid (5) and fumaric acid (6).

With the exception of chickpea samples, in which no phenolic compounds were detected, phenolic acids and flavones were the metabolites identified in GL seeds. The profile quietly varied between species and in some cases within varieties of the same species. Samples with a higher content and a more detailed profile in phenolic compounds were field peas (0.15-0.44 mg g^{-1} DM) and faba beans (0.30-0.41 mg g^{-1} DM).

Conclusions

Although for some species, PT varieties were not as proteinaceous as F ones, they all represent good sources of protein, energy and unsaturated fatty acids for humans and animals. The content of xantophylls, citric acid and phenols is indicative of the antioxidant power of these seeds in biological systems. Oxalates do not constitute a problem in any of the samples studied.

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MODELLING THE TEMPERATURE AND pH DECLINE EARLY POST-MORTEM OF BEEF CARCASSES

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Abstract

The objective of this work was to model the pH and temperature decline early post-mortem on beef carcasses and to study the effect of gender, genotype and weight class on the pH and temperature decline patterns. A total of 24 beef animals slaughtered in a local abattoir were sampled. pH and temperature were recorded using an OMEGA wireless receiver/host (UWTC-REC1). The decline of pH and temperature was modelled using one parameterisation of the exponential decay function, and its parameters were estimated using the software R. The fitted models were used to predict pH and temperature at 1.5 h, at 3.0 h and at 24 h; the time when pH reached 6.0, and the temperature at which pH reached 6.0. The rate parameters of the exponential decay function for pH (KpH) and temperature (KT) were found to be independent ($r=0.35$, $P>0.05$). The correlation between pH at 3 h and final pH (at 24 h) was very high ($r=0.930$, $P<0.01$). The KT was influenced by the time elapsed from slaughter until the first recording, and by the carcass weight. In opposition, those variables did not affect the KpH. The exponential decay function was able to model the early post-mortem decline of both pH and temperature, and the pH at 3 hours can be used as predictor of the final pH of beef meat.

Keywords: pH, temperature, decline, meat, exponential decay

EFFECTS OF SALINITY CHALLENGE ON ION REGULATION IN ESTUARINE MIGRATING SEA LAMPREY, *PETROMYZON MARINUS*.

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Abstract

The sea lamprey, Petromyzon marinus, is an anadromous species in which the adults re-enter freshwater, and migrate upstream for terminal spawning. A reduction in salinity tolerance has been document in migrants although the underlying mechanisms have not been characterized. The aim of this study was to determine the capacity for marine osmoregulation in late, upstream migrants by characterizing the morphological and physiological effects of salinity challenge from a molecular perspective. Salinity was gradually raised until a final salinity of 17.5‰. A number of relevant blood and intestinal parameters were measured to assess ionoregulatory and biochemical changes as well as the expression of key ion-transport related proteins by immunoblotting (IB) [Na+/K+-ATPase (NKA), vacuolar-type H+-ATPase, carbonic anhydrase, and Na+:K+:2Cl-contransporter]. NKA activity was also measured, in addition to oxidative stress indicators.

The upper lethal salinity limit was determined to be > 17.5‰ in the freshwater migrating adult lamprey. At 17.5‰ lamprey displayed a clear osmoregulatory failure, being unable to regulate Na+ and Cl- levels with plasma and intestinal fluid approaching environmental concentrations (osmoconforming and failure of drinking mechanism, respectively). This was accompanied by a significant drop in hematocrit (37% to 1%) and plasma lactate concentrations indicating hemolytic anemia. Higher plasma [ALT] indicated tissue damage that correlates with oxidative damage to liver (high lipid peroxidation and GST activity, and lower [GSH]). No ion transport protein response to salinity was detected in gill, intestine or kidney by immunoblotting. The only potentially adaptive ionoregulatory response was an increase in Na+/K+-ATPase activity in mid-intestine.

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YOUNG OF THE YEAR (YOY) FLOUNDER CONDITION AND RELATIONSHIP WITH HABITAT FEATURES IN THE LIMA ESTUARY (NW PORTUGAL)

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Abstract

In nursery areas optimal habitat characteristics, such as high prey availability and low predation, promote enhanced fish growth and condition. Small scale variability of environmental parameters throughout an estuarine nursery may be responsible for differential spatial and temporal patterns in fish density and condition. The northern coast of Portugal represents the southern distribution limit of the flounder *Platichthys flesus*. Environmental changes may increase the vulnerability of juvenile flounder in this area, resulting in poorer condition affecting fish recruitment. These aspects were investigated in the Lima estuary (northern Portugal) using spatial and temporal patterns of YOY, flounder condition, and its relationship with habitat features. Flounder juveniles and their main prey (macroinvertebrates) as well as environmental data were collected monthly between May and October, in 2010 and 2013. Generalized additive models (GAM) relating flounder condition (Fulton's *K*) to habitat characteristics showed that sediment and water characteristics shaped spatial patterns of YOY flounder condition, with potential effects on growth, survival and recruitment to adult populations. The improved knowledge of habitat features promoting YOY flounder condition in its distribution limit will help prioritize areas for conservation and efficient management.

FUTURE DIRECTIONS IN FISH NUTRITION

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Abstract

Over the past decades we have recorded a more than thirty-fold increase in aquaculture production. There are also previsions that show that by 2050, this growth of aquaculture will lead to a situation where the total volume of seafood production (fisheries + aquaculture) will be above that of any individual terrestrial animal production: beef, poultry or pork. Since capture fisheries are stable with no prospects of any foreseeable increase, this will have to come from aquaculture. The major responsibility of the actors of today is to make sure that that this growth in fish production is managed in a sustainable manner. As of today, fish farming at the global level is still dominated by freshwater species from relatively low trophic levels. But, there are clear indications that "feed-based aquaculture" will increase with the consequence of an increasing demand for nutritionally complete feeds. There is also great concern as regards the reliance of fish and shrimp feeds on fish meal (FM) and fish oil (FO) derived from capture fisheries. Although the volume of wild fishery resources diverted for feed purposes has not increased over the past decades and there are clear indications that they have in fact declined over the past decade. Research in the area of replacement of FM and FO as dietary sources of essential amino acids, fatty acids and micronutrients has also made much progress in the recent years and is reflected by the significant reductions in the inclusion of these ingredients in the commercial feeds. Ensuring the nutritional value of farmed fish as food for man is another issue which will continue to need our attention. The potential consequences of the use of feeds with low marine ingredients over the full life cycle, from larvae to broodstock, of different species also will need more attention. With the increasing contribution of aquaculture to the human food basket, the demand for nutrients for farming fish and shrimp will rise, competing with other animal production sectors. This growth is also subject to competition for direct or indirect land and water use. There is a strong demand for the assessment of the environmental footprint of different farming systems and aquaculture products. Indeed, more than in terrestrial animal production systems, interactions between aquatic animals and the environment can have greater consequences, assessment of which at the farm or at the global level will need novel approaches and methods. Improving the efficiency of resource (nutrient, energy, water, land) utilization will make aquaculture sustainable, where nutrition research has a strong role to play.

Fish nutrition research has recognized the importance of optimal nutrient and energy supply for improving productivity in aquaculture and have made efforts to update our knowledge on the nutrient requirements of several fish and shrimp. But, compared to terrestrial livestock production relying on a relatively small number of species across the world, aquaculture involves the farming of a large number of species in a wide variety of environments and farming systems. There is a need for precision-farming approach of all aquatic organisms all through their life cycles. As a science and practice, fish nutrition research has a great responsibility to make the sustainable development a reality, by judiciously exploiting emerging ever novel tools and concepts.

USE OF PROBIOTICS INTERCROPPED WITH PLANT PROTEIN DIETS AND THEIR INFLUENCE ON THE GROWTH PERFORMANCE AND IMMUNOLOGICAL STATUS OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

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Abstract

In Senegalese sole (*Solea senegalensis*) aquaculture, infectious diseases represent a major challenge, leading to significant economic losses (Arijo et al, 2005). According to FAO / WHO (2001), probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host". Previous studies working with different fish species provided evidence on the effectiveness of various probiotic bacteria in promoting the fish welfare (Castex et al., 2009; Hernandez et al., 2010; Merrifield et al., 2011).

Keywords: Probiotics, immune status, plant protein sources, growth performance, Senegalese sole

In the current study, we evaluated the effects of dietary probiotic supplementation intercropped with plant protein as fishmeal replacement on the growth performance and immunological status in sole (*Solea senegalensis*). Fish were fed for 10 weeks on six isonitrogenous and isolipidic diets (55% protein and 8% lipid, dry matter basis), formulated to have 35% or 72% of fishmeal replacement by plant protein (PP_{35} or PP_{72}) intercropped with three probiotic supplementation (NO – unsupplemented, PRO_1 and PRO_2). The probiotics were tested at a dosage recommended by the manufacturer. PRO_1 was a multi-species probiotic bacteria (*Bacillus sp.*, *Pediococcus sp.*, *Enterococcus sp.*, *Lactobacillus sp.*) supplemented at 1.8×10^{10} CFU kg^{-1} diet (CFU - colony forming unit) and PRO_2 was an autolyzed yeast (*Saccharomyces cerevisiae*) supplemented at 4g kg^{-1} diet. Zootechnical parameters were measured at start and the end of the feeding trial for growth performance determination. Plasma was collected at 3, 17, 38 and 73 days of feeding dietary treatment for the determination of innate immune response (plasma lysozyme and peroxidase contents, plasma alternative complement pathway activity – ACH50) in order to detect differences between treatments as well to identify the influence of long or short-time probiotic supplementation.

Fish with an initial body weight of 33.1 ± 0.2 g grew to a maximum final body weight of 50.6 ± 1.2 g. PRO_1 groups had significantly lower weight gain and higher feed conversion ratio (11.9 ± 1.9 and 2.2 ± 0.4, respectively) compared to NO groups (17.4 ± 1.9 and 1.5 ± 0.1 respectively). Growth performance from PP_{35} and PP_{72} groups did not differ, suggesting that sole can cope well with diets containing high plant protein levels, as already reported by Silva et al. (2009).

At 3 and 73 days of feeding trial, the immune parameters analysed did not present differences among treatments, indicating that these may not be the most appropriate

sampling times to detect the influence of probiotic administration in Senegalese sole. Plasma peroxidase content (EU mL^{-1}) was not affected by PP level, but was significantly changed by probiotic administration at 38 days of feeding. PRO_1 groups presented higher plasma peroxidase content (79.3 ± 18.6) comparing to PRO_2 groups (40.1 ± 10.3), but were not statistically different from NO groups (57.2 ± 14.0). Plasma lysozyme content (EU mL^{-1}) and ACH50 (Units ml^{-1}) were not affected by probiotic administration. However, fish fed PP72 diets had higher lysozyme and ACH50 levels at 38 days (1018 ± 204 and 257 ± 21, respectively) than fish fed PP35 diets (646 ± 154 and 183 ± 17, respectively). Also at 17 days, fish fed PP72 diets had higher lysozyme (345 ± 82) comparing to fish fed PP35 diets (175 ± 37).

In conclusion, the results of our study suggested that high plant protein inclusion levels may affect Senegalese sole immune status, without affecting growth performance. Multi-species probiotic (PRO_1) was more effective in stimulating the humoral innate immune parameters than *Saccharomyces cerevisiae* (PRO_2). Further studies are needed to evaluate whether dietary probiotic supplementation may induce higher resistance against stress conditions and pathogens in sole.

Acknowledgements

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Silva J.M.G., Espe M., Conceição L.E.C., Dias J., Valente L.M.P. 2009. Senegalese sole juveniles (*Solea senegalensis* Kaup, 1858) grow equally well on diets devoid of fish meal provided the dietary amino acids are balanced. *Aquaculture*, 296, 309–317. DOI:10.1016/j.aquaculture.2009.08.031

DEVELOPMENT AND ESTABLISHMENT OF AN EFFECTIVE AND REPRODUCIBLE INFECTON MODEL OF *TENACIBACULUM MARITIMUM* AGAINST SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

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Introduction

One of the most significant pathogen that threatens Senegalese sole (*Solea senegalensis*) aquaculture is the Gram-negative bacterium *Tenacibaculum maritimum*, the main etiological agent of marine tenacibaculosis. Like other filamentous bacteria, *T. maritimum* presents many obstacles during laboratory work, resulting in a tendency for aggregation, fluctuation and adhesion to all kind of substrates thus revealing difficulties in detection and recognition of the colonies. The colonies usually exhibit active spreading (swarming) and polymorphism, making enumeration of the bacteria by classical methods impossible. Nowadays, there is no evidence for establishment of an effective challenge models as the bacterium presents some constrains during its culture. Moreover, there is a considerable lack of knowledge regarding the mechanisms of infection and the interactions between this particular pathogen and the host. The main goals of this study were to optimize proper culturing conditions for *T. maritimum* in order to draw standard growth curves, and to develop an effective and reproducible challenge model using different bacterial strains.

Material and methods

Three *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) isolated by Prof. Alicia Toranzo (Spain) from Senegalese sole in a local fish farm (Póvoa de Varzim, Portugal) were used during experimental assays. Molecular identification of bacterial strains was done according to (1). Several bacterial growth trials were performed using marine broth (MB) supplemented with several chemical reagents as follows: Non-ionic surfactant (Sigma) with different concentrations (0.1%, 1%, 10% and 100%) and cellulose hydrolysis were used according to (2). Sodium dodecyl sulphonate was also added at different concentrations (1%, 5% and 10%; Sigma). Another bacterial culture approach was performed by inoculating the bacterial strains directly into MB with no other reagents added and with strong aeration. Bacteria were cultured in marine agar (MA) during 48h and inoculated in MB according to the above conditions at 25ºC and continuous shaking. Growth curves were obtained after recording optic density readings (600 nm) and bacterial counting using a Helber count chamber every 2h during 72h.
One hundred and eight acclimated Senegalese sole without history on tenacibaculosis were divided into three equal groups each of 36 fish. Each group was reared in a separate recirculation seawater system (tª 23-25°C; 80% O_2 saturation; salinity 32; and natural photoperiod) comprised by six tanks with 6 fish per tank. Each group was inoculated with three final concentrations of each *T. maritimum* strain in duplicate ($2.6 \times 10^{5-7}$, $9.6 \times 10^{5-7}$, $4.8 \times 10^{5-7}$ for ACC6.1, ACC13.1 and ACC20.1, respectively) by a prolonged bath method: 24 h with strong aeration. Another group of 36 fish was inoculated with sterile saline solution under the same conditions and served as control. Experimentally infected fish were monitored daily and mortalities recorded during 15 days. Dead fish were sampled for bacteriological assessment. Bacterial colonies were identified using

morphological, physiological and biochemical tests according to (3) and confirmed by conventional and nested PCR as previously described elsewhere (1).

Results and discussion

Among the different approaches employed with media supplementation, the non-ionic surfactant with 100% concentration did not reveal bacterial fluctuation. However, relative low growth was obtained compared to that obtained after direct inoculation in MB and strong aeration, which was selected to study *T. maritimum* growth. This approach showed exponential growth for all bacterial strains, which was equivalent to 4.9×10^8, 4.8×10^8 and 4.85×10^8 cfu/ml for ACC6.1, ACC13.1 and ACC20.1, respectively. Maximum bacterial growth was achieved after 48 h incubation period while the lag phase started at 50 h at which the bacterial concentrations and numbers began to decrease. The experimental infection of Senegalese sole by different strains of *T. maritimum* was successfully conducted through the prolonged bath method. Most fish died during the first week in case of ACC6.1 and ACC20.1 strains while fish exposed to ACC13.1 extended for 15 days when exposed to the highest concentration. Different lethal dose 50 (LD50) were obtained depending on the strain: 2.6×10^5, 9.6×10^5 and 4.8×10^5 cfu/ml for strains ACC6.1, ACC13.1 and ACC20.1, respectively. Thus, bacterial strains ACC6.1 and ACC20.1 exhibited a great similarity in their pathogenicity whereas ACC13.1 appears to be less virulent. Interestingly, all bacterial strains used in experimental infection belong to serotype O3. All infected fish showed skin ulcerations mainly in the dorsal side, tail rots, red mouth and fin erosions (Fig. 1A). Internally, all fish exhibited pale gills, friable livers and severe congestion in kidneys (Fig. 1B). Regarding bacteriological assay, all bacterial strains were successfully isolated from external lesions and kidneys on MA and FMM after 48 h incubation period. Samples from external and internal (mainly kidney) injuries revealed positive results for conventional and nested PCR. Results from the present study provide a new approach for tenacibaculosis induction without preliminary trials for gill and skin abrasion. Hence, these findings will be instrumental to study the pathogenicity of tenacibaculosis as well as the host/pathogen interactions, which are not clearly understood yet.

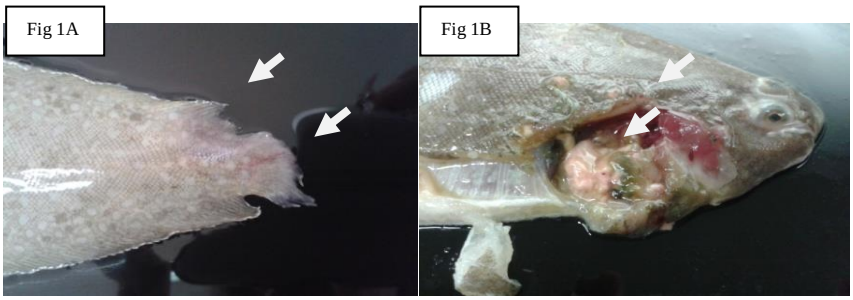

Fig. 1. Experimentally infected Senegalese sole with *Tenacibaculum maritimum* showing sever tail and fin erosions (A) and friable liver and congested kidney (B).

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EFFECT OF SEAWEED SUPPLEMENTATION ON GROWTH PERFORMANCE, IMMUNE AND OXIDATIVE STRESS RESPONSES IN GILTHEAD SEABREAM (*SPARUS AURATA*)

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Abstract

Seaweed composition include several bioactive compounds with nutraceutical properties. The dietary seaweed supplementation in aquafeeds may give a competitive edge over unsupplemented fish to cope to stressful conditions under aquaculture intensive conditions. The current study was designed to evaluate the effects of seaweed supplementation on growth performance, immune and oxidative stress responses in gilthead seabream (*Sparus aurata*). Three seaweed species, each from one of the following groups, Rhodophyta (R), Phaeophyta (P), Chlorophyta (C), and a mix of all three (M) were supplemented to the experimental diets at 2.5% and 7.5% (R2.5%, P2.5%, C2.5%, R7.5%, P7.5% and C7.5%, M7.5%) and tested against a control unsupplemented group (CTRL). The M7.5% diet was supplemented with 2.5% of each of the three seaweed. A total of 360 gilthead seabream juveniles, 31.2 ± 0.6g were allocated in 24 rectangular tanks, 115 L capacity each, with a 4L/min water renewal, and fed twice a day until satiation for 90 days. Growth performance was significantly improved in all seaweed treatments, mainly daily growth index was 14.4% higher than control. Immune response did not differ among the dietary treatments. The digestive enzyme amylase showed higher activity in all 7.5% supplemented diets with about 2.27 times fold increase. Glutathione oxidized showed significant differences to supplemented treatments with an average 44% lower. Glutathione reductase, glutathione s-transferase, glutathione peroxidase and catalase tended increase to diets with seaweeds. Results indicate seaweed supplementation improved growth and fish welfare, even if not significantly, nonetheless a favorable tendency to supplemented treatments results was disclosed.

Keywords: Seaweed; Algae, *Sparus aurata*; Gilthead seabream; Nutraceutical compounds.

Acknowledgments:
This research was supported by project ALGADOURADA (31-03-05-FEP-41), funded by program PROMAR, which in turn was partially supported by the European Fisheries Fund (EEF). *This research was also partially supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the project "PEst-C/MAR/LA0015/2013.*

PROTEIN HYDROLYSATES AFFECT PROTEIN ACCRETION AND THE REGULATION OF MUSCLE GROWTH IN SENEGALESE SOLE LARVAE

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Abstract

Fish nutrition research has suggested that the partial substitution of dietary native proteins by highly hydrolysed fish meal would result in better larval perfomances. In the last years, this hypothesis has been studied and different experimental and commercial hydrolysates differing in the raw material have shown remarkably different effects on larval gut maturation and/or growth. However possible transcriptomic effects underlying such response have not been explored so far. To our knowledge, no studies have been published regarding the effect of feed composition and/or nutritional programming on fish larvae metabolic capacity and muscle growth *via* possible epigenetic mechanisms.

In the present study, we hypothesized that a 40% substitution of native proteins by a commercial fish protein hydrolysate (HYDROL diet) delivered from mouth opening onwards would positively influence Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis with possible consequences on muscle development and long-term growth. The diet based on native proteins (INTACT) was shown to promote a higher absorption of smaller peptides at the metamorphosis climax (17DAH), compared to the HYDROL diet. The INTACT diet has also promoted a higher proliferative capacity of myogenic cells at both stages, as it increased the percentage of small fibers (∅<5µm). However, at 36DAH the HYDROL diet upregulated the transcript levels of *Myogenin (myog),* which encodes a highly conserved myogenic regulatory factor that is involved in terminal muscle differentiation. This could possibly indicate some attempt of compensatory growth. These results suggest that the inclusion of highly hydrolyzed protein in the diet does not promote protein utilization or growth at later stages of sole development.

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POSTERS

USE OF PROBIOTICS IN SOLE (*SOLEA SENEGALENSIS*) DIET: EFFECTS ON NUTRITION PERFORMANCE, HOST DEFENSE, MORPHOLOGY AND ECOLOGY OF THE DIGESTIVE TRACT

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Keywords: Probiotics, immune status, plant protein sources, growth performance, gene expression, intestine histology, Senegalese sole,

Abstract

In sole (*Solea senegalensis*) aquaculture, infectious diseases represent a major challenge, leading to significant economic losses. Conventionally, antibiotics are the most used products for the treatment of bacterial diseases. However, sick fish eat less food and simultaneously pathogenic drug-resistant limit their use. Probiotic is defined as a body or substance which contributes to the proper balance of microorganisms in the digestive tract, promoting their immune defenses and nutritional/ physiological development. Experiments with different fish species provided evidence of the effectiveness of various probiotic bacteria in promoting the fish well-being, especially in situations of biotic and abiotic stress (e.g. environmental and nutritional sub-optimal conditions).
In this project, we intend to assess the nutritional, immunological and molecular genetics basis of the protective effects of probiotic agent in sole (*Solea senegalensis*) nutrition. For that purpose we have designed 3 experiments to study the effect of probiotic use on: a) Growth performance and food utilization; b) Immune status characterization (innate immune parameters and gene expression); c) Intestinal morphology and ecology; and d) Protective effect after stress conditions.
The 1st experiment aimed to determine the effects of dietary probiotic supplementation on growth, gut morphology and non-specific immune parameters in Senegalese sole (Solea senegalensis) juveniles during a 1-month trial. Fish were fed for 1 month two diets with 1.0 or 4.6 × 10^6 CFU kg^{-1}; CFU - colony forming unit) of probiotic A (*Bacillus* sp., *Pediococcus* sp., Enterococcus sp. and Lactobacillus sp.) and two diets with 3.5 or 8.6 × 10^5 CFU kg^{-1} of probiotic B (*Pediococcus acidilactici*), and tested against an unsupplemented diet (control). Growth performance, as well as respiratory burst activity (ROS), nitric oxide (NO), alternative complement pathway (ACH50), lysozyme and peroxidase activities were not affected by the dietary treatments. Probiotic supplementation tended to increased growth homogeneity between tanks having diet A_1 the best possible alternative to decrease costs associated to size grading. Villous length and number of goblet cells of the anterior intestine did not vary among treatments. Muscle duodenal layer was significantly thicker in fish fed probiotic A compared to probiotic B, when included at the lowest level (A_2 *vs* B_2). The current study indicate that the use of the multi-specie probiotic at 1.0 × 10^6 CFU kg^{-1} might enhance protection against pathogen outbreak and increase nutrient absorption, whereas at the highest concentration could reduce size dispersion among tanks. From this experiment one poster presentation (XV International Symposium on Fish Nutrition and Feeding – ISFNF 2012) was achieved and one paper was accepted to publication (Aquaculture Nutrition - Doi: 10.1111/anu.12191).
The 2nd experiment aimed to evaluate: a) the effects of dietary probiotic supplementation intercropped with the use of plant protein as fishmeal replacement on the humoral innate immune parameters and related transcript levels in sole (*Solea*

senegalensis); and b) histological and microflora changes in sole fed plant protein diets intercropped with the use of probiotics. Six isoproteic and isolipidic diets (55% protein and 8% lipid, dry matter basis) contained a plant protein level of 25% or 75% (CTRL or PP) and one of three probiotic supplementation (NO – unsupplemented, PRO_1 and PRO_2), were tested during a 73 days trial. PRO_1 was a multi-species probiotic bacteria (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) supplemented at 1.8×10^{10} CFU kg^{-1} diet) and PRO_2 was autolyzed yeast (*Saccharomyces cerevisiae*) supplemented at 4g kg^{-1} diet. Zootechnical parameters were measured at the start and the end of the feeding trial for growth performance determination. At 73 days of feeding trial, plasma was collected for the determination of the humoral innate immune responses (plasma lysozyme and peroxidase contents and plasma alternative complement pathway activity – ACH50). A segment from the foregut was collected for histology evaluation (villous length, muscular layer thickness and goblet cells). Also hindgut, rectum and head-kidney samples were collected to determine the mRNA transcript levels of some immune related genes (lysozymes – *lyzc* and *lyzg*; heat shock proteins – *hsp90a*, *hsp90b* and *hsp90b1*; iron chelating proteins – *ftm* and *apoa1*; complement factors – *c3a* and *c3b*; the cysteine protease *casp3*; and oxidative enzymes – *cat* and *gpx*). Our results suggest that plant protein based diets may affect immune gene expression of Senegalese sole, despite not affecting growth performance. However, in our study, diets not supplemented with probiotic showed to have better growth performance. At the gene expression, all tissues analyzed were significantly affected by plant protein content (hindgut - *hsp90b* and *apoa1*; rectum - *casp3*, *gpx* and *cat*; head-kidney - *hsp90b1*), presenting PP diets lower values. Also probiotic supplementation had significantly affect hindgut gene expression (*hsp90b*, *hsp90b1* and *gpx*) and rectum gene expression (*hsp90a*, *ftm* and *apoa*). Histology data are still in calculation and statistical analyse. From this experiment two oral presentation (International Meeting on Marine Research 2014 and Aquaculture Europe 2014) were achieved and two paper are being written to future publication.

A 3rd experiment was delineated with the purpose to determine the use of autochthonous bacteria of sole (*Solea senegalensis*) intestine as a potential probiotic and their effect on welfare and growth after a pathogen challenge. This experiment will be inserted on an AQUAEXCEL project and will start at October 2014. There are 3 different main point differing from the other 2 experiments: 1) the use of open-water flow system; b) The use of two potential probiotic bacteria isolated from *Solea senegalensis* intestine; and c) study the probiotic effect on fish post a Challenge trial.

As preliminary conclusions, we may say that probiotic use on sole diets could affect growth performance in an ambiguous way by increasing growth homogeneity but also decreasing growth performance. Also duodenum histology (mucus layer thickness) was changed with type of probiotic used during experiment. Diets with high content of vegetables ingredients have proven to stimulate the sole innate immune response. And we may also say that the probiotic change intestinal micro flora of Senegalese sole. However, the beneficial effects of probiotics still remain controversial and further studies should address the mechanism of action involved in the protection against stress conditions (nutritional, environmental, infections by pathogens).

Acknowledgements

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IDENTIFICATION OF A NOVEL CARBONIC ANHYDRASE ISOFORM IN LARVAL LAMPREY (*PETROMYZON MARINUS*)

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Abstract

Carbonic anhydrase (CA) plays a key role in CO_2 excretion and acid-base regulation in vertebrates. The lamprey is a basal vertebrate and to date only a single CA isoform has been identified and characterized although multiple isoforms are found in other vertebrate groups. Lampreys have a complex life cycle in which the larvae or ammocoetes are benthic filter feeders which undergo a dramatic metamorphosis into parasitic feeding juveniles. In the case of *Petromyzon marinus*, the post-metamorphic juveniles migrate to the sea to commence feeding.

The aim of this study was to determine if additional branchial CA isoforms are present in *P. marinus* gill at different life history stages. A RT-PCR approach was taken using degenerate and consensus sequence primers with samples from adult, ammocoete and post-metamorphic transformer stages. A novel partial sequence was identified in ammocoete gills and the full length sequence was obtained by RACE PCR. The new ammocoete CA transcript is 1786bp coding for a 257 aa protein that has 67.5% aa identity with the published CA isoform from lamprey and 56.3% and 57.4% for teleost and mammalian CAc and CA2 isoforms, respectively. Phylogenetic analysis indicates that the ammocoete CA isoform clusters basally within the cytosolic CA clade. Higher transcript expression was found in ammocoete compared to transformer gill. Western blotting using a heterologous cytosolic CA antibody with gill tissue from different ammocoete stages and transformer indicates a pronounced shift in CA size, supporting the hypothesis of changes in branchial isoform expression during metamorphosis. Supported by FCT PTDC/MAR/98035/2008.

USING MUSCLE CELLULARITY, TRACER STUDIES AND GENE EXPRESSION TO UNDERSTAND THE EFFECT OF DIETARY PROTEIN COMPOSITION ON PROTEIN DIGESTIVE CAPACITY AND MUSCLE GROWTH

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Abstract

There is evidence that prenatal nutritional supply affects skeletal muscle development in a variety of vertebrates, with possible long-term consequences on growth potential and flesh quality. The amino acid (AA) imbalance is one of the causative factors for increased catabolism and possible loss of some essential AA to energy catabolism and other metabolic purposes. The dietary protein content and quality, may therefore, determine fish larvae capacity to digest protein and utilize it for muscle growth. In particular, the availability of certain amino acids seems to play a major role in stimulating protein accretion and skeletal myofibre numbers. However, the knowledge about the influence of early nutritional programming on muscle development and growth is still rudimentary in fish. Even more unclear are the mechanisms underlying such possible influence.
For that reason we decided to investigate whether the dietary protein composition could influence skeletal muscle cellularity and growth in a fish species that undergoes a complex metamorphosis. In Senegalese sole larvae fed inert diets from mouth opening, increased dietary protein content showed a positive effect on larval growth from 2 to 51 DAH overcoming the effect of an imbalanced AA profile. However, no major influence was found on the larvae capacity to digest and retain protein. Although the proliferative capacity of myogenic cells and the growth potential of Senegalese sole larvae remained unaffected at the metamorphosis climax (19 DAH), at juvenile stage (51DAH), the dietary protein composition did affect muscle cross-sectional area. The expression levels of growth-related genes such as the myogenesis regulatory factors (MRFs) were studied in an attempt to highlight the main physiological mechanisms through which the dietary protein affected muscle growth during the metamorphosis climax and at the juvenile stage.

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EFFECT OF SEAWEED SUPPLEMENTATION ON GROWTH PERFORMANCE, IMMUNE AND OXIDATIVE STRESS RESPONSES IN GILTHEAD SEABREAM (*SPARUS AURATA*)

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Abstract

Seaweeds could be an excellent source of nutraceutical compounds, offering more capability to cope to stressful conditions and minimizing disease outbreaks. Furthermore, seaweed is part of the natural diet of several marine fish species, with potential palatability properties for aquaculture diets. The current study was designed to evaluate the effects of seaweed supplementation on growth performance, immune and oxidative stress responses in Gilthead Seabream (*Sparus aurata*). Three seaweed species, each from one of the following groups, Rhodophyta (R), Phaeophyta (P) and Chlorophyta (C), were supplemented to the experimental diets at 2.5% and 7.5% and tested against a control diet (Ctrl) with no supplementation. In addition to six diets with seaweed in two different levels of supplementation: R2.5%, P2.5%, C2.5%, R7.5%, P7.5% and C7.5%; a diet Mix, supplemented at 7.5% (2.5% of each algae) was also tested. A total of 360 Gilthead Seabream fingerlings, 13.07 ± 0.13cm and 31.17 ± 0.63g were allocated in 24 rectangular tanks, 115L capacity each, with a 4L/min water renewal, and fed twice a day until satiation. Monitored water parameters encompass: temperature, dissolved oxygen, salinity, ammonia and nitrites, all maintained within the species wellness conditions. At day 90 of the trial a final sampling was made. Length, weight, blood, liver and gut samples were collected to analyze growth performance: specific growth rate and feed conversion ratio; immunologic parameters: lisozyme and peroxidase; digestives enzymes: amylase, chimiotripsin, tripsin and lipase; oxidative stress: lipid peroxidation, glutathione S-transferase, glutathione peroxidase, glutathione reductase, total glutathione, glutathione oxidized, acetilcholinesterase and catalase. Growth performance parameters showed significant difference, about all supplemented treatments than control. In terms of the immunology, there is no signicant difference. Regarding digestive enzymes, only amylase showed higher activity to supplemented diets. Oxidative stress showed significant difference only to glutathione oxidized, but glutathione reductase, glutathione s-transferase, glutathione peroxidase and catalase tended increase to diets with seaweeds. Results indicate seaweed supplementation improved growth and fish welfare, even if not significantly, nonetheless a favorable tendency to supplemented treatments results was disclosed.

Keywords: Seaweed; Algae, *Sparus aurata*; Gilthead seabream; Nutraceutical compounds.

Acknowledgments:

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GRAIN LEGUMES PRODUCTION UNDER RAINFED CONDITIONS IN PORTUGAL

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Introduction

European Union is deficient in vegetable protein sources, being strongly dependent on the import of protein sources (mainly oilseeds and their meals) from third countries. Several measures have been purposed to increase grain legumes (GL) production in European countries, namely in Portugal (Report A7-0026/2011, 2011). Despite the fact that Portugal presents potential to produce GL, the actual area occupied by these crops is limited due to 1) a focus of agriculture production on winter cereals, 2) no use of legumes in rotations and 3) low yields (≤1000 kg/ha). The aim of this work was to review the state of knowledge on the GL production in Portugal focusing on varieties of chickpeas, field peas, faba beans and lupins, as these are important GL in Mediterranean farming systems (Carranca *et al.*, 1999) and of interest, among others, to the feed industry.

Edaphoclimatic conditions

Portugal is a Mediterranean country with wide precipitation variability, periodic droughts and sudden and intensive downpours. In Entre-Douro e Minho (EDM), Beira Litoral (BL), Trás-os-Montes (TM), and Beira Interior (BI) prevails a temperate climate with dry or temperate summer, and in Estremadura e Ribatejo (ER), Alentejo (Alj), and Algarve prevails a temperate climate with dry/hot summer; in a small region of Alentejo, a cold steppe climate can also be observed. Generally, Portuguese soils are of poor agricultural suitability as they are highly erodible, present medium to low cation exchange capacity, a pH below that considered optimal for plant growth and low levels (~ 1%) of organic matter (REA, 1999).

Nitrogen fixation

Grain legumes establish symbiotic relationships with rhizobacteria, being able to fix N_2 and to store it in root nodules. Values of N_2 fixed by GL in national territory are reported on Table 1 and are in accordance with data of other Mediterranean countries. Chickpea was found to fix less N_2 (20-60 kg N_2 ha/year; Kumar and Abbo, 2001).

Table 1. Nitrogen fixation by grain legumes in Portugal.

Grain legume	N_2 fixed (kg/ha/year)	Agricultural region	Reference
Faba bean	76-125	Alentejo	Carranca *et al*. (1999)
Field pea	31-107	Alentejo	Carranca *et al*. (1999)
White lupin	>100	Ribatejo	Carranca *et al*. (2009a)
Yellow lupin	89	Trás-os-Montes	Castro (1999)

Sowing season

In Mediterranean conditions, faba beans, field peas and lupins are sown in autumn/winter while chickpea is traditionally sown in spring due to the lack of resistance to the fungus *Ascochyta rabiei* (however, resistant varieties have been selected). Portuguese studies on sowing season of GL reported that autumn sowing increased 1) grain yield in chickpea (Figure 1), field pea and faba bean, 2) plant habit in chickpea allowing for mechanical harvesting and 3) seeds weight in field pea (Costa Pinto *et al.*,

1990; Barroso *et al.*, 2007). Lupins are also reported elsewhere to get higher yields in autumn sowing (Mut *et al.*, 2012).

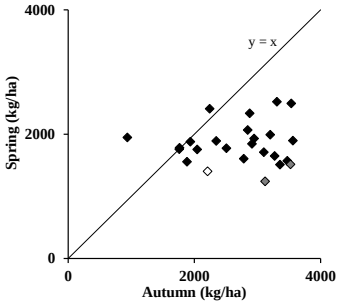

Figure 1. Effect of sowing season on grain yield of chickpeas.

The results presented suggest clear advantages of autumn comparing to spring sowing. Indeed, constrains related with delayed sowing are high temperatures/sun irradiation and irregular/scarce rainfall, leading to shortening of growing cycle, low and irregular yields, unsuitable plant habit and consecutively need of manual harvesting.

Multi-site yield experiments

Yield experiments in autumn sowing under rainfed conditions were already performed with national and foreign GL varieties in different Portuguese agricultural regions. Grain yields reported, both for national and foreign varieties, are above those traditionally observed. With more than 2000 kg/ha, chickpea production seems also viable in TM, in addition to ER and Alj, where it is usually sown. Field pea yields above 3000 kg/ha in all agricultural regions suggests an adaptation to a wide range of agronomic conditions. Low grain yields of white lupins in Alj – comparatively to TM and BI – is probably related to Alj low fertile, sandy and acidic soils, once lupins are more demanding in terms of soil conditions. Higher yields found for bitter yellow lupins explains why Portugal largely resort on them. However, sweet yellow lupins showed great performances in TM.

Cereal-based rotations

In Portugal, rainfed cereals are sown in autumn/winter in N poor fields, commonly in rotation with fallow (not recommended). Rotations with GL can greatly contribute to the development of the following cereal by restoring the N/other nutrients in the soil and decreasing the incidence of pests/diseases in cereals and GL, constituting more economic and sustainable farming practices. In TM, Castro (1999) found higher yields and N levels of cereal (wheat and triticale) grain and straw when in rotation with yellow lupin, rather than in a cereal-fallow system. Carranca *et al.* (2009b), in ER, reported an increase in oat biomass when preceded by white lupin comparatively to a continuous oat-oat.

Conclusions

Several varieties of chickpea, field pea, faba bean and lupins (Portuguese or not) are well-adapted to our edaphoclimatic conditions, capable of being sown in autumn and grown under rainfed conditions, with final grain yields above those traditionally observed. Grain legumes-based rotations, replacing the traditional fallow, could benefit the cereal and contribute to the development of organic and more extensive farming systems, leading to higher farmer incomes while helping to combat human desertification in North and Central inland, and South (Alentejo) of the country.

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