PROBIOTIC SUPPLEMENTATION PROMOTES CALCIFICATION IN *Danio rerio* LARVAE

A MOLECULAR STUDY

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Abstract

- Recent studies show that dietary probiotics supplementation can have beneficial effects on both humans and animals. It was demonstrated that probiotic *Lactobacillus rhamnosus* - a component of the human gut microflora, enhances reproduction, larval development, and the biomineralization process in *Danio rerio* (commonly known as Zebrafish).
- If *Danio rerio* larvae is treated with probiotic *L. rhamnosus*, then the *L. rhamnosus* may influence the host's development, opening new prospects for probiotic use and their applications. This was demonstrated through the study with regards to the results obtained.
- The aim of this study was to identify the pathways affected by *L. rhamnosus* during zebrafish larval development. The morphological and histochemical findings show that *L. rhamnosus* accelerates bone deposition through stimulation of the expression of key genes involved in ossification, e.g: runt-related transcription factor 2 (*runx2*), Sp7 transcription factor (*sp7*), matrix Gla protein (*mgp*), and bone gamma-carboxyglutamate (*gla*) protein (*bglap*) as well as through inhibition of sclerostin (*sost*) - a bone formation inhibitor.
- The results and molecular findings obtained showed that in zebrafish larvae treated with *L. rhamnosus*, presented developmental changes; induction of osteoblast and osteocyte differentiation evidenced by the expression of key genes involved in ossification. Acid-free double staining demonstrated changes that happen during larval development due to probiotic supplementation. Other gene expression changes were identified after real-time PCR analysis. Western blot analysis of mitogen-activated protein kinase 1 and 3 (Mapk1 and Mapk3), which are involved in osteoblast and osteocyte differentiation showed an increase in Mapk1 16 days post fertilization (dpf) and of Mapk3 at 23 days post fertilization (dpf) in individuals receiving *L. rhamnosus* supplementation.
- Probiotic administration activated the expression of the genes involved in osteoblast differentiation and bone formation which caused the changes in the development of *L. rhamnosus* treated larvae. This suggests that the probiotic can have effects on the *Danio rerio* larvae.

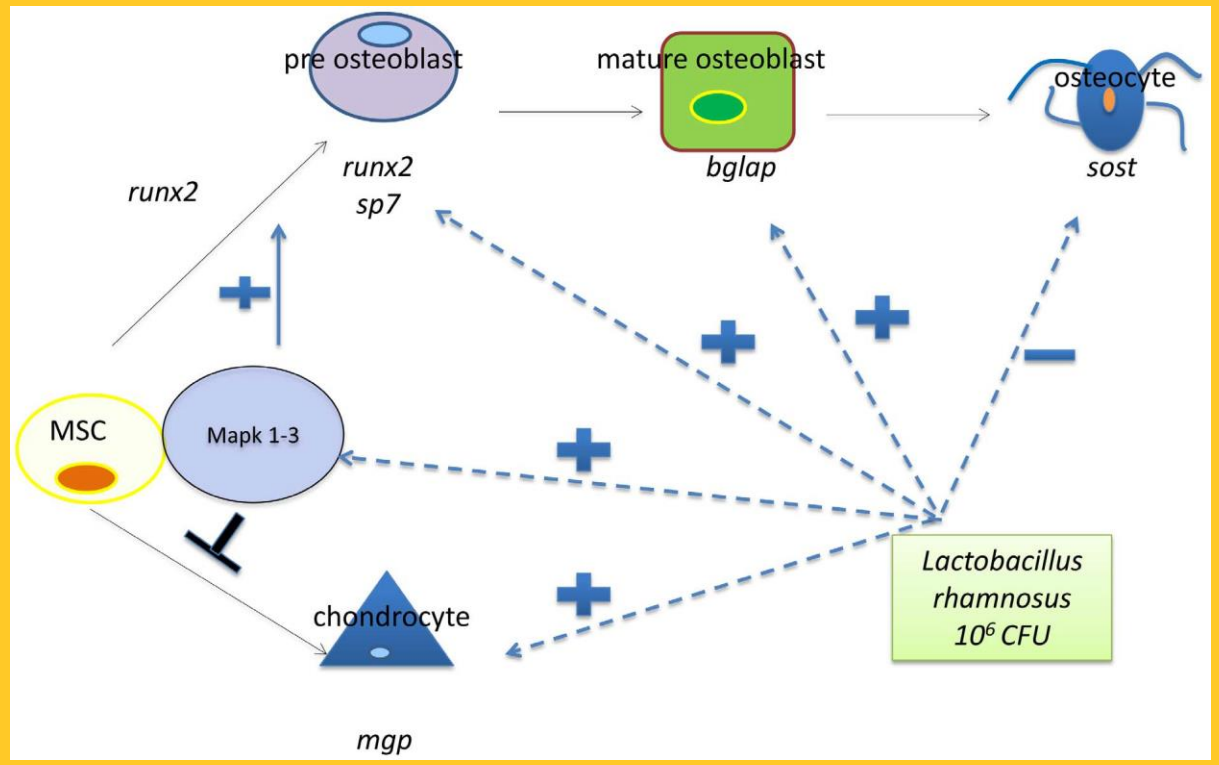
Riassunto
Studi recenti dimostrano che l'integrazione alimentare di probiotici può avere effetti benefici sia sull'uomo che sugli animali. E' stato dimostrato che il poriobiotico Lactobacillus rhamnosus - un componente della microflora intestinale umana, è in grado di migliorare la riproduzione, lo sviluppo larvale e il processo di biomineralizzazione in *Danio rerio* (comunemente noto come pesce zebra). Lo scopo di questo studio era di identificare i percorsi regolati da L. rhamnosus durante lo sviluppo larvale di zebrafish. Le analisi morfologiche e istochimiche mostrano che L. rhamnosus accelera la formazione dell'osso stimolando l'espressione dei geni chiave coinvolti nell'ossificazione come ad esempio: fattore di trascrizione correlato a runt 2 (runx2), fattore di trascrizione Sp7 (sp7), proteina di matrice Gla (mgp) e proteina gamma-carbossiglutammato (gla) ossea (bglap) nonché attraverso l'inibizione della sclerostina (sost) - un inibitore della formazione ossea. L'analisi Western blot delle protein chinasi 1 e 3 attivata da mitogeno (Mapk1 e Mapk3), coinvolte nella differenziazione degli osteoblasti e degli osteociti, ha mostrato un aumento della Mapk1 16 giorni dopo la fecondazione (dpf) negli individui che ricevevano l'integrazione di L. rhamnosus. Lo studio ha anche mostrato una riduzione dello sclerostin (sost) in individui che assumevano L. rhamnosus suggerendo un ruolo positivo del probiotico nei trattamenti di disturbi ossei. Attraverso tecniche molecolari e istologiche come la doppia colorazione, sono stati identificati cambiamenti che si verificano durante lo sviluppo larvale a causa della supplementazione di probiotici. La Real Time PCR ha messo in evidenza cambiamenti di espressione genica di geni coinvolti nel processo di ossificazione

Introduction

- Lately, interest in the benefits of probiotic supplementation on teleost health , immune function, stress tolerance and development has significantly augmented. There are studies that demonstrated that chronic administration of *L. rhamnosus* may influence the larval development of Danio rerio (zebrafish).
- In this study alcian blue-alizarin red double staining demonstrated that probiotic administration accelerates skeletal formation in *Danio rerio* (zebrafish) larvae, and real-time PCR documented changes in the expression of a set of key genes involved in bone formation.
- The cells involved in zebrafish bone formation and remodeling are similar under many respects to those of mammals. The master genes of osteoblast differentiation are *Runx2* and *Sp7*. *Runx2*, expressed in early osteoprogenitors, induces the gene expression program required for mesenchymal stem cells (MSC) lineage determination and differentiation and is also required for osteoblast function after differentiation. *Runx2* is expressed in the early development stages of numerous cell types, e.g. chondrocytes. Two *runx2* orthologs have been identified and characterized in zebrafish; *runx2a* and *runx2b* are both expressed in developing skeletal elements and show differences in their expression patterns.
  Sp7 is thought to be involved in the regulation of numerous osteoblast genes including osteocalcin, osteonectin, osteopontin, and collagen type I. Molecular studies have demonstrated that *Bglap* accumulates in the extracellular matrix of mammalian mineralized bone and its expression is specific to bone tissue and dentin, whereas *Mgp* is mainly associated with cartilage, soft tissue and the vascular system. The role of sclerostin (*sost*) in the pathogenesis of sclerosis and in the onset of bone disease has been intensively investigated showing that has a negative role as a regulator of bone formation in the aging skeleton.
- Osteoblasts respond to and differentiate as a consequence of two main factors: chemical signals and physical stress. These stimuli activate specific signaling pathways such as MAPK (mitogen-activated protein kinase). The two major MAPK isoforms are Mapk1 and Mapk3. The two proteins are co-expressed in virtually all tissues, albeit with quite a variable relative abundance. Mapk1 and Mapk3 both play an important role in osteoblast differentiation.

Osteoblast differentiation in zebrafish larvae

- Osteoblasts are responsible for bone formation, and impaired osteoblast development leads to serious bone diseases such as osteoporosis. Hedgehog (Hh) signaling and autophagy are two important regulators of bone differentiation.
- Hedgehog (Hh) has the ability to induce ectopic cartilage and bone formation and stimulates osteoblastic differentiation mainly through up-regulation of *Sp7* and *Runx2* gene expression in osteoblastic cells.
- During osteoblast differentiation, rapid synthesis of bone matrix protein results in accumulation of misfolded proteins, and high autophagy activity is thus required for their removal. Hedgehog (Hh) signaling pathway suppresses autophagy level in zebrafish larvae. Inhibition of autophagy induces osteoblast related genes (*Sp7*, *Runx2*, *Mgp*, and *Bglap*) up-regulation in transcription and protein levels, and promoted osteoblast mineralization.



It is demonstrated in the figure that in *L. rhamnosus* treated larvae, Sost gene have a negative role as a regulator of bone formation.
Sp7, *Runx2*, *Mgp* and *Bglap* genes are expressed in *L. rhamnosus* treated larvae, playing a key role as a regulators of osteoblast differentiation.
Mapk1 and Mapk3 are co-expressed in *L. rhamnosus* treated larvae, both playing an important role in osteoblast differentiation.

Figure 1. Proposed model of *L. rhamnosus* role in osteoblast differentiation

Results and discussion

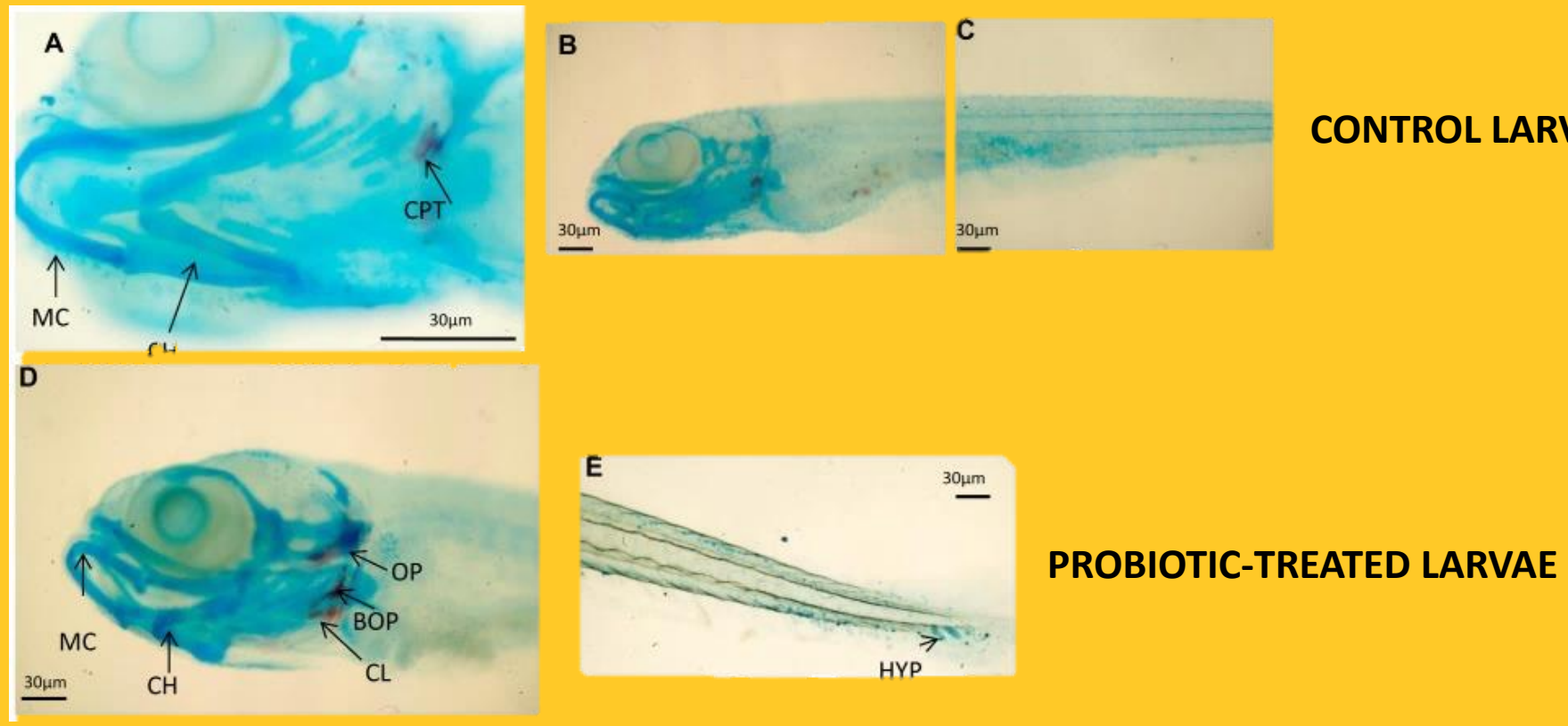


Figure 1. Skeletal development in zebrafish using alcian blue-Alizarin red double staining.
A = 9 dpf zebrafish control larvae head skeleton presenting calcified pharyngeal teeth (CPT)while other structures like Meckel's cartilage (MC) and ceratohyal (CH) remain as cartilage.
[B-C] = 9 dpf control zebrafish head (B) and trunk (C) presenting no signals of bone calcification.

D = A 9 dpf *L. rhamnosus* fed zebrafish larvae head skeleton presenting calcification of the opercula (OP), cleithrum (CL) and basioccipital articulatory process (BOP). Meckel's cartilage (MC) and ceratohyal (CH) remain as cartilage;
E = 9 dpf *L. rhamnosus* fed zebrafish presenting the first hypurals (HYP) developing.

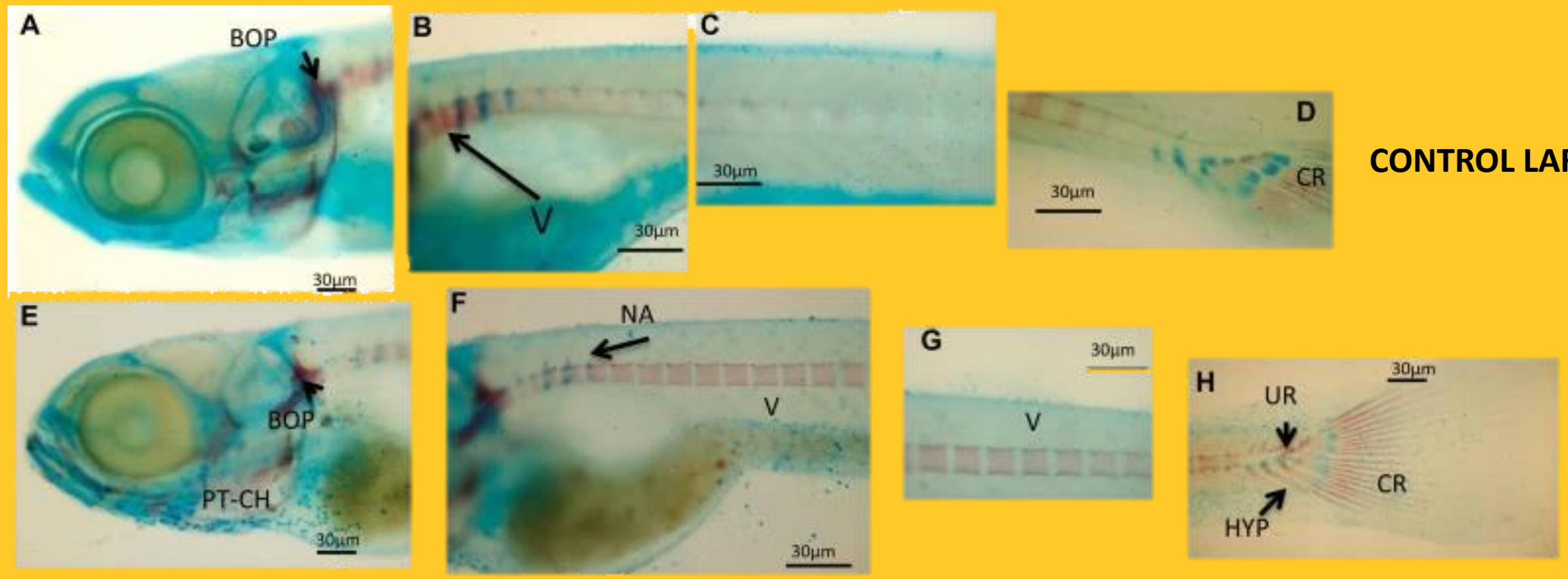


Figure 2. Double staining of skeleton in larvae sampled at 16 dpf.
[A-D] Images showing significant aspects of skeleton development in control zebrafish larvae.
B = Formation of first vertebrate (V).
D = Caudal hypuralia acquires final number of structures with modified hemal arches and caudal fin rays (CR).

[E-H] Representative images showing the development of the skeleton in zebrafish fed *L. rhamnosus*.
E = Prescence of calcified pharyngeal teeth and ceratohyal (PT-CH)
F-G = Vertebrae formation (in an anterior posterior direction) toward the posterior end of the notochord. Formation of the first neural arches (NA) is observed dorsally in the anterior vertebrae.
H = Beginning of calcification of the hypural (HYP) under the urostyle (UR) and presence of calcified caudal fin rays.

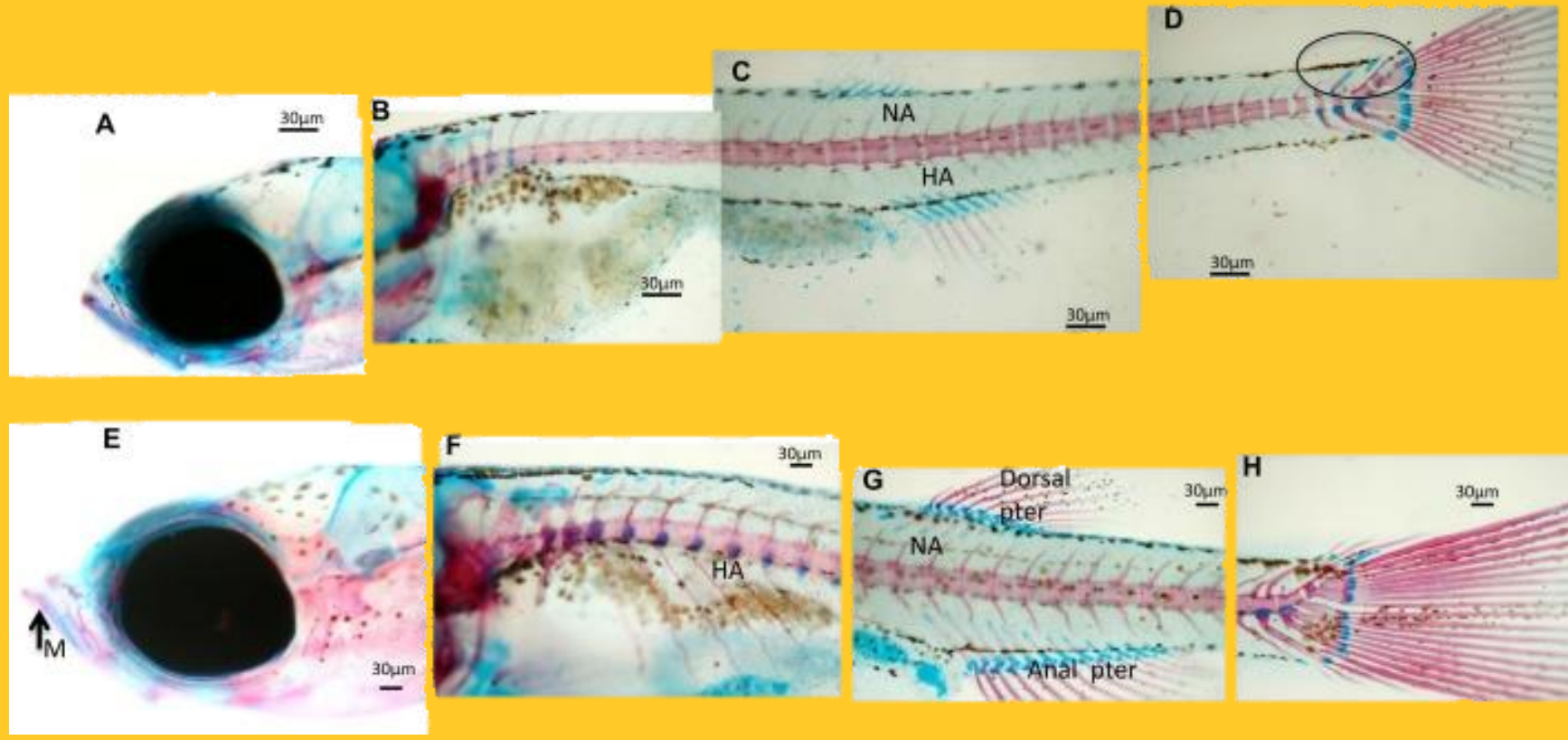


Figure 3. Double staining of the skeleton in larvae sampled at 23 dpf.
[A-D] Images showing significant aspects of skeleton development in control zebrafish larvae.
C = Shows neural arches (NA) and sketches of hermal arches (HA) evidently.
Caudal skeleton still presents cartilagenous structure evidenced by a circle

[E-H] Representative images showing the development of the skeleton in zebrafish fed *L. rhamnosus*.
E = Presence of calcified mandibular (M).
Neural arches (NA) and hemal arches (HA) are detected in the whole trunk of the larvae.
G = Complete formation of dorsal and anal pterygium.
H = Caudal skeleton is complete.

Molecular findings on gene expression

A = *runx2* gene expression did not vary significantly at the different time points in control larvae (C), whereas in probiotic specimens (P) its levels were significantly increased at 23 dpf compared with 9 and 16 dpf and with control individuals.

B = *sp7* expression rose significantly in control larvae (C), showing at 23dpf a 30-fold increase compared with 9 dpf levels.
In probiotic larvae (P), sp7 expression peaked at 16 dpf and at 23 dpf reverted to 9 dpf levels.

C = *mgp* gene transcripts showed lower levels at 23 dpf compared with 9 and 16 dpf in both groups; however levels were significantly higher in probiotic (P) vs. control (C) zebrafish at 9 and 16 dpf and similar at 23 dpf.

D = *bglap* expression peaked at 23 dpf and was significantly greater in probiotic larvae.

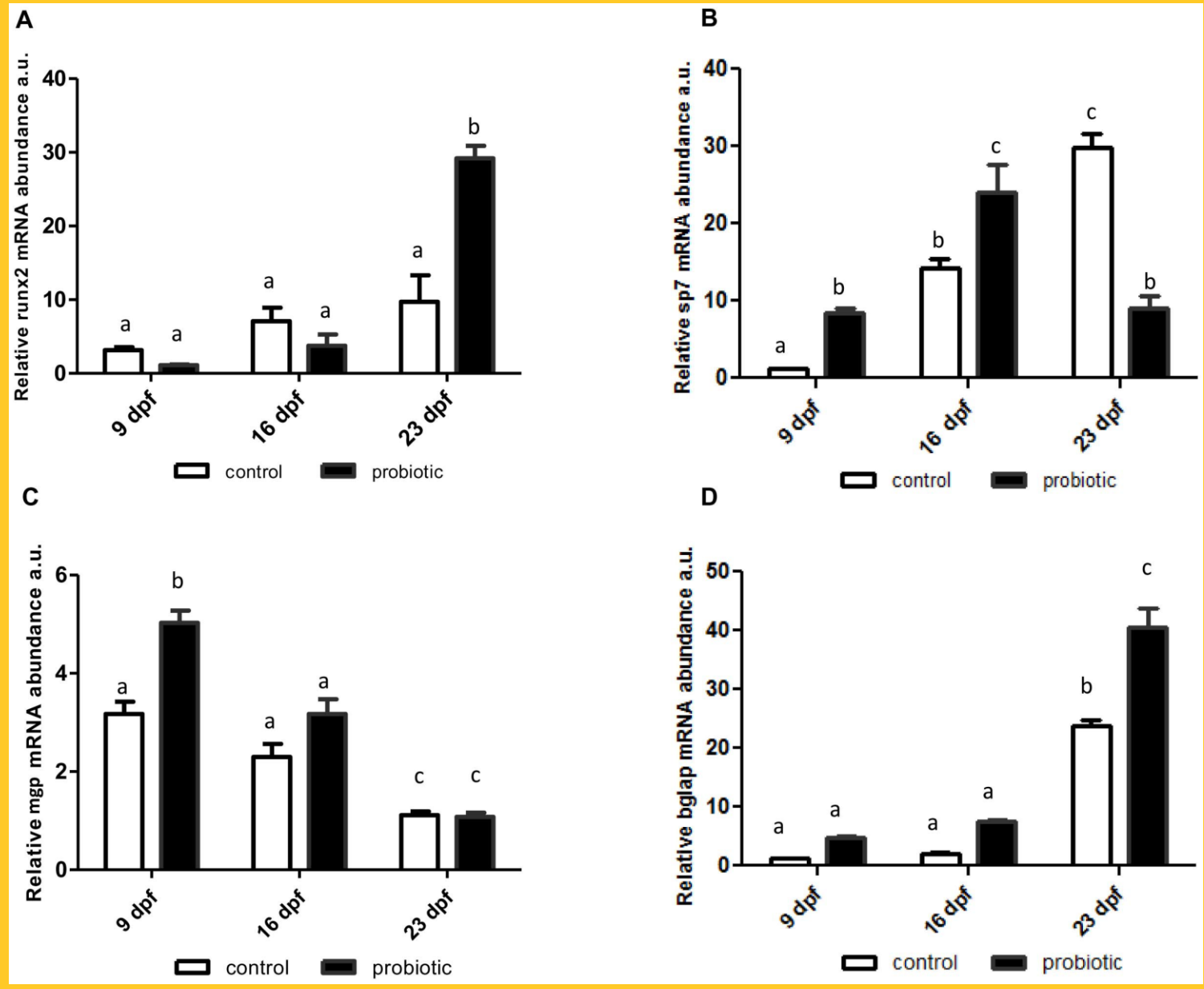


Figure 4. *runx2* (A), *sp7* (B), *mgp* (C), *bglap* (D) mRNA levels.

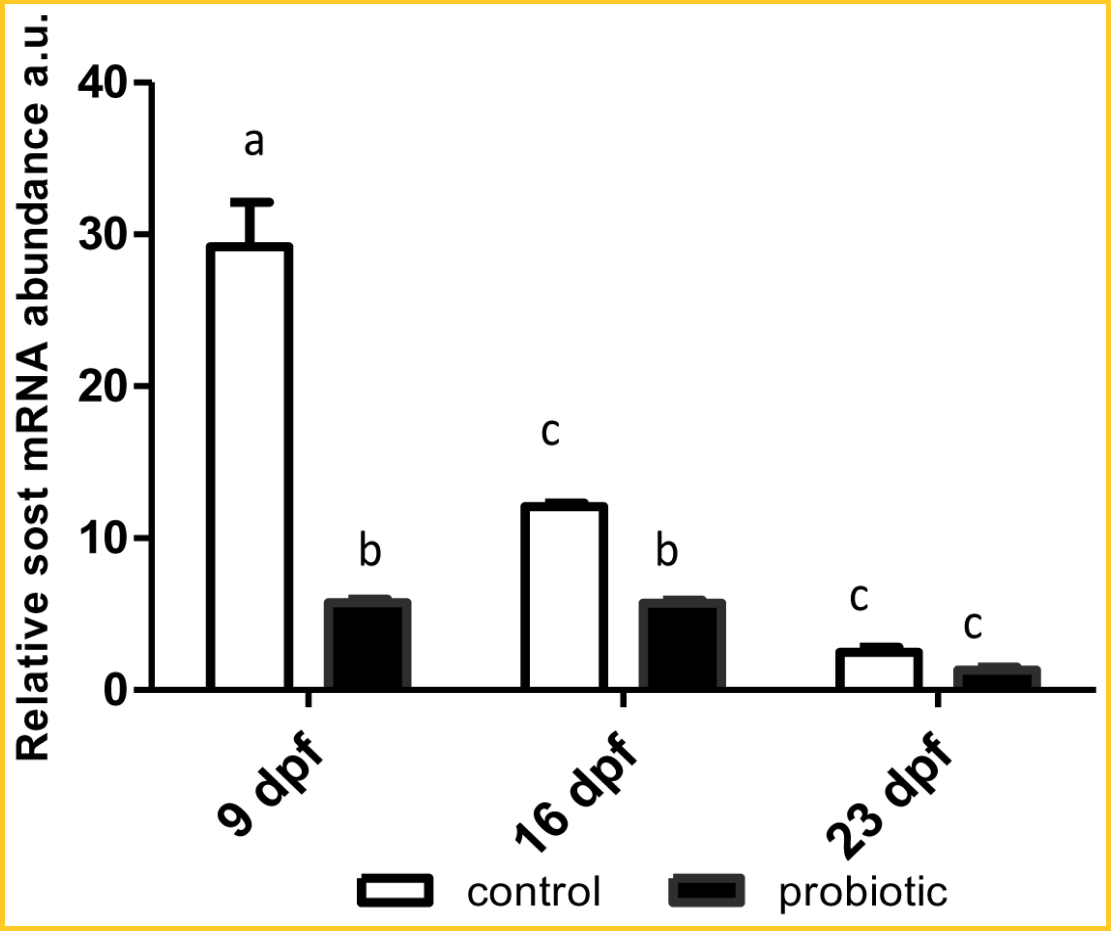


Figure 5. *sost* mRNA levels.
sost expression was peaked at 9 dpf, then declined reaching the lowest level at 23 dpf.
The expression was higher in control larvae at 9 and 16 dpf whereas at 23 dpf there were no differences between control and probiotic larvae.

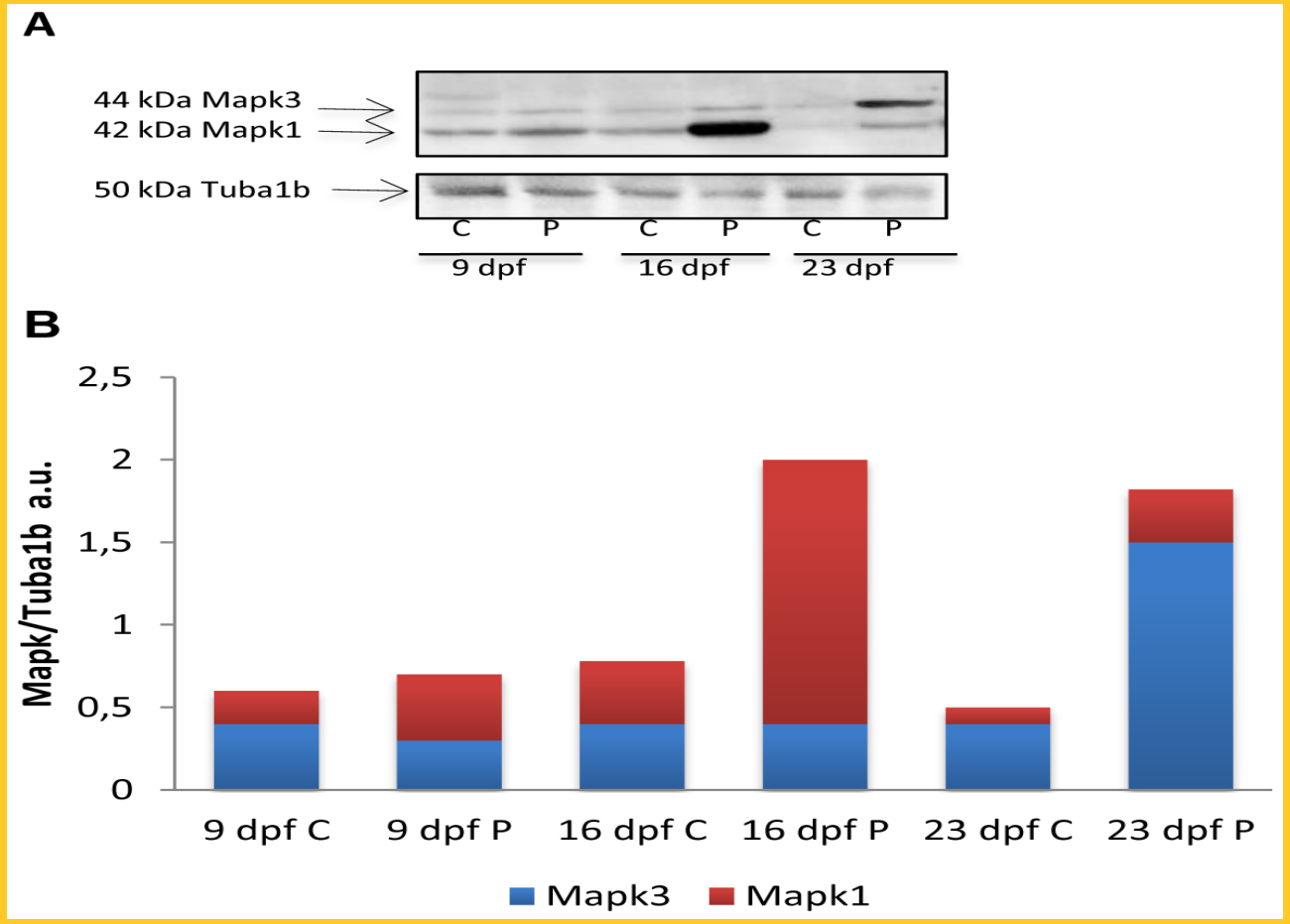


Figure 6. Mapk1/3 Western blot.
A = Representative Mapk1/3 and Tuba1b in control and probiotic treated fingerlings sampled 9, 16 and 23 dpf.
B = Densitometric analysis
Mapk1/3 antibody cross-created with a doublet of the expected molecular weight (44-42 kDa) and showed similar levels in control and probiotic specimens at 9 dpf and an increase in Mapk1/3 protein levels at 16 and 23 dpf in P larvae.

Conclusion

- Through morphometric evaluation and histochemical staining (von Kossa and alcian blue/alizarin red) with the analysis of the expression of *Runx2* and *Sp7*, the genes involved in early osteoblast differentiation and bone formation the differences in the control larvae and the probiotic-treated were shown during *D.rerio* development.
- The *Runx2* and *Sp7* were up-regulated in the treated group, suggesting enhanced bone formation in these animals. The mgp upregulation observed in probiotic-treated zebrafish confirms that these fish are in a more advanced stage of development compared with controls. *Mgp* gene expression and protein accumulation reflect the patterns of formation of cartilaginous and mineralized structures.
- *Sost* expression was highest in control animals at 9 dpf and gradually declined until 23 dpf. Sost encodes sclerostin, a protein synthesized by osteocytes that can down-regulate osteoblast formation. Its inhibition results in increased bone production, leading to the notion that compounds that reduce its levels could be harnessed to treat osteoporosis and other skeletal disorders.
- At the molecular level, the treated group had higher levels of Mapk1/ 3, which may participate in the regulation of genes involved in osteocyte formation.
- Since zebrafish have been established as a vertebrate model for biomedical research, the present findings provide data for the use of *L. rhamnosus* as a support to human treatment.

References

1. Maradonna F, Gioacchini G, Falcinelli S, Bertotto D, Radaelli G, et al. (2013) Probiotic Supplementation Promotes Calcification in Danio rerio Larvae: A Molecular Study. PLoS ONE 8(12): e83155. doi:10.1371/journal.pone.0083155
2. Rawls F, Samuel BS, Gordon JI (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc Natl Acad Sci USA 101:4596–4601.