Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN SILICO APPROACH FOR THE IDENTIFICATION AND CHARACTERISATION OF BIOACTIVE PEPTIDES FROM SILVER CARP COLLAGEN

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Abstract

The paper aimed to identify in silico bioactive peptides with antioxidant and antihypertensive effects from silver carp collagen. This approach involved the use of a wide range of specialized online databases and tools to identify bioactive peptides from various protein sources. In this case, the collagen type-I alpha-1 protein sequence was extracted from UniProtKB with the identification code A0A077B3P8. The digestion was simulated using the BIOPEP database with the following enzymes: subtilisin, papain and pepsin. ExPASy ProtParam, Peptide Ranker, PepCalc, and ToxinPred showed that silver carp collagen is a significant source of biologically active peptides, health promoters with potential antihypertensive and antioxidant effects. The computational approach used in this study offered useful initial insights for more extensive studies.

Key words*: bioactive peptides, collagen, health effects, in silico, silver carp,*

INTRODUCTION

By-products, generated from industrial fish processing represent over 50% of the total weight of the source, and their market value is low. Without processing, such by-products transform into dangerous wastes due to intrinsic microbiological instability. These residues sourced from skin, bones, viscera, scales, heads or fins are rich in collagen, which is a potential source of bioactive compounds applicable in the pharmaceutical, biomedical, cosmetic, and food industries (Pati et al., 2010; Pal & Suresh, 2016).

Until recently, collagen from various animals, such as bovine or porcine components, was preferred as a source of potential compounds with health-promoting effects. Due to various consumer perception issues, such as potential prion contamination of animal collagen, or ethical considerations, alternative sources of collagen have begun to be sought (Karim & Bhat, 2009).

Collagen derivatives from various sea sources are possible health promoters through various antihypertensive, antioxidant, neuroprotective, and regulatory effects. These effects result from bioactive collagen compounds such as bioactive peptides released when collagen interacts with proteolytic enzymes (Pal & Suresh, 2016). Due to the high content of amino acids such as glycine and proline in the structure of carp collagen, its potential for the generation of bioactive peptides is high (Pal & Suresh, 2017).

To the best of our knowledge, data on the biological activities of the bioactive peptides that could be generated from silver carp collagen are limited in the literature. This study explores the use of possible health-promoting agents of type I alpha-1 collagen in silver carp (*Hypophthalmichthys molitrix*) released at the interaction with various proteolytic enzymes. Using *in silico* approach, we attempted to predict the possible bioactive peptides with antihypertensive (angiotensin-converting enzyme/ACE inhibitors) and antioxidant activities when collagen is subjected to enzymatic hydrolysis. Also, we focused on the release from fish carp collagen of the peptides

with sensory characteristics, such as umami taste (Zhao *et al*., 2019). Collagen is an appropriate substrate to release umami peptides due to its high content in glutamic and aspartic acids (Gan et al., 2022).

MATERIALS AND METHODS

We used the alpha-1 type I collagen protein sequence from the silver carp (*H. molitrix*) that we acquired from the UniProt database (http://www.uniprot.org; UniProt entry: A0A077B3P8_HYPMO), accessed on 10.09.2021. To check the amino acid content of type I alpha 1 collagen in silver carp, for the entry mentioned above from UniProt, we used the Expasy ProtParam online tool (https://web.expasy.org/protparam/) (Gasteiger et al., 2005).

We chose the option "Profiles of potential biological activity" in the "Analysis" section of the BIOPEP instrument (http://www.uwm.edu.pl/biochemia/index.php/en/biopep; accessed on 10.09.2021), to predict the different profiles of the silver carp collagen protein potential biological activity. This option generated a list with various health-promoting effects, depending on the appearance of bioactive fragments in alpha-1 type I collagen chains in proteolysis simulation. The results obtained here included information such as ID, name, activity, number, sequence, and location of the peptide in the protein sequence. To check the potential of type I alpha-1 collagen protein as a possible substrate generating peptides with antihypertensive and antioxidant activities, the "Calculations" option was selected from the "Bioactive peptides" menu of the BIOPEP instrument. The result generated here is based on the equation: A=a/N, where A=frequency of occurrence of bioactive peptides, a=number of bioactive peptides, and N=total number of amino acid residues in the chosen protein sequence (Minkiewicz et al., 2019).

For the *in silico* prediction of the theoretical peptide sequences generated when collagen interacts with various proteolytic enzymes, the program "Enzyme (s) action", available in the BIOPEP instrument, was used. The enzymes used for protein proteolysis in this study were: subtilisin (EC 3.4.21.62), papain (EC 3.4.22.2), and pepsin (pH>2) (EC 3.4.23.1). The protein of interest, i.e., type I collagen from the silver carp (*H. molitrix*) was subjected to the simulation of enzymatic hydrolysis individually with each enzyme and a mix of two enzymes: pepsin+papain. The peptides obtained at this step were subjected to the "Search for active fragments" option, and the antihypertensive and antioxidant peptides were selected and extracted for further analysis.

The bioactive potential of ACE inhibitory and antioxidant peptides selected at the previous step was predicted using the online tool PeptideRanker. (http://bioware.ucd.ie/~compass/biowareweb/). This server can predict if a peptide is bioactive, generating peptide scores between 0-1, where a score close to 1 means high bioactivity (Pooja et al., 2017).

All the theoretical peptides obtained at the *in silico* proteolysis step, were subjected to the bioactivity test, after which they were manually included in the top 5 of the most bioactive peptide sequences.

To predict *in silico* theoretical parameters of peptides such as molecular weight, isoelectric point, or solubility of peptides in water, the peptides obtained in the previous step were subjected to prediction tests using PepCalc (https://pepcalc.com/) and ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) programs, the latter predicting possible toxicity of peptides (Pooja et al., 2017).

The prediction of the sensory characteristics of bioactive peptides obtained from type I alpha-1 collagen in silver carp was performed using the BIOPEP database. The number of bitter and umami-taste peptides resulting from the collagen protein, was calculated using the "Enzyme(s) action" option in the "Sensory peptide and amino acids" menu of the BIOPEP instrument (Iwaniak et al., 2016).

RESULTS AND DISCUSSIONS

Regardless of the origin, type I collagen contains about 20 different amino acids, which are found in different proportions and achieve the triple helix conformation due to their organization in three α chains, which wrap around each other (Shoulders & Raines, 2009).

Of these amino acids, glycine represents the highest percentage, about 27%, and

hydroxyproline and proline together about 25% of the total amino acids, which explains the repetitive Gly-X-Y sequence, where glycine is found at every third residue (Gauza-Włodarczyk et al., 2017; Coppola et al., 2020). Such repetitive sequence is directly related to the triple helix highly packed secondary structure. The high content of amino acids such as alanine, proline, and hydroxyproline is characteristic of type I collagen (Jafari et al., 2020)

Using the Expasy ProtParam program, we found varying amounts of amino acids in the silver carp protein sequence. Table 1 shows the amounts of amino acids according to the COL1A1 sequence extracted from the UniProtKB database.

Table 1. The amino acid content of type I alpha 1 collagen in silver carp (*H. molitrix*) according to Expasy ProtParam (https://web.expasy.org/protparam/)

Aminoacid	Acronym 3- letter*	Acronym 1- letter*	Amount (%)
Alanine	Ala	A	10.10%
Arginine	Arg	R	4.70%
Asparagine	Asn	N	2.10%
Aspartate	Asp	D	4.40%
Cysteine	Cys	B	1.20%
Glutamine	Gln	C	2.80%
Glutamate	Glu	E	5.50%
Glycine	Gly	Q	26.80%
Histidine	His	Z	0.60%
Isoleucine	Ile	G	2.30%
Leucine	Leu	H	2.60%
Lysine	Lys	I	4.10%
Methionine	Met	L	1.70%
Phenylalanine	Phe	K	2.00%
Proline	Pro	M	17.20%
Serine	Ser	F	3.90%
Threonine	Thr	P	4.30%
Tryptophan	Try	S	0.40%
Tyrosine	Tyr	T	0.70%
Valine	Val	W	2.60%

*IUPAC-IUB Commission on Biochemical Nomenclature - Rules, 1968

Table 1 shows glycine as the most abundant amino acid, with about 26%, followed by proline at 17%, alanine at 10%, glutamic acid at 5%, aspartic acid, and arginine threonine at 4%, serine at 3%, and methionine about 1.7%. These data are in agreement with the findings of other researchers in the analysis of type I collagen from various sources (Shoulders & Raines, 2009; Gauza-Włodarczyk et al., 2017; Song et al., 2017; Czerniecka-Kubicka et al., 2020).

The BIOPEP database contains a wide range of online tools that can be used in the predictive analysis of bioactive peptides in various protein chains. At the time of accessing the BIOPEP database, it contained a range of 740 proteins and 4199 sequences of bioactive peptides. It is important to note that in similar attempts to generate bioactive peptides from the same type of silver carp collagen protein used in this study, the results may be different, as the database may undergo changes in sequence content (Minkiewicz et al., 2019).

Table 2. Profile of the potential biological activity of type I alpha-1 collagen in silver carp (*H. molitrix*)

	Activity	Frequency (A)
1	dipeptidyl peptidase IV inhibitor	0.8273
2	ACE inhibitor	0.8087
3	antithrombotic	0.2079
4	regulating	0.1906
5	antiamnestic	0.1899
6	dipeptidyl peptidase III inhibitor	0.0725
7	antioxidative	0.0566
8	inhibitor	0.0456
9	alpha-glucosidase inhibitor	0.0407
10	chemotactic	0.0325
11	stimulating	0.0090
12	renin inhibitor	0.0076
13	neuropeptide	0.0055
14	activating ubiquitin-mediated proteolysis	0.0041
15	bacterial permease ligand	0.0028
16	CaMPDE inhibitor	0.0028
17	embryotoxic	0.0021
18	immunostimulating	0.0014
19	HMG-CoA reductase inhibitor	0.0007
20	hypolipidemic	0.0007
21	immunomodulating	0.0007

For alpha-1 type I collagen protein, our analyses showed the existence of several profiles of potential biological activity. The predicted significant potential biological

activity was dipeptidyl peptidase IV inhibitory activity with an activity frequency of A=0.8273, followed by inhibitory ACE activity with A=0.8087, antithrombotic activity A=0.2079, regulatory activity A=0.1906, anti-amnestic (Alzheimer preventive) activity A=0.1899 and the inhibitory activity of peptidyl peptidase III (protease involved in the modulation of peptide hormones signaling) A=0.0725. The antioxidant activity was ranked seventh with an activity frequency of A=0.0566 (Table 2).

Pal and Sureh (2016) researched an ACE inhibitory activity frequency of A=0.7011 and A=0.7993 for dipeptidyl peptidase IV inhibitory activity at *in silico* simulation of type 1 alpha-1 collagen from *Ctenopharyngodon idella*. The antioxidant activity found by researchers for this type of collagen was A = 0.0546 (Pal & Suresh, 2017).

Our study is in agreement with the data obtained by other researchers, type I alpha-1 collagen showing potential release effects of peptides with different bioactive roles, which places this protein as a potential candidate for the development of new products with antihypertensive, antioxidant, antidiabetic, or antithrombotic effects.

The number of bioactive peptides with ACE inhibitory and antioxidant activities obtained at the *in silico* simulations can be observed in Figure 1.

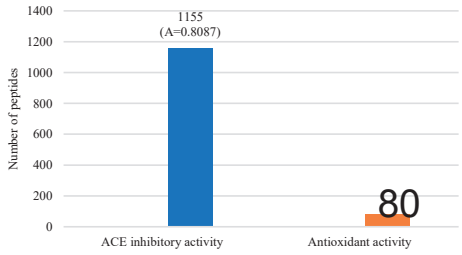

Figure 1. Prediction of the number of potential bioactive peptides in silver carp collagen showing the value of parameter A

The potentially significant biological activity was observed in the case of inhibitory ACE activity, with a number of 1155 potential bioactive peptides and a value of the quantitative parameter A=0.8087, compared to the antioxidant activity with 80 peptides obtained and A=0.0566. The value of the quantitative parameter A shows the ability of the protein to release bioactive peptides. Therefore, a higher value of this parameter means a higher possibility that a specific activity will be predominant (Minkiewicz et al., 2019).

Data on potential bioactive peptides resulting from type I alpha-1 collagen in silver carp are limited. The present work is among the first studies to demonstrate that this type of low-cost protein, with high accessibility, is a possible generator of peptides with antihypertensive and antioxidant activities, using an *in silico* approach.

Figure 2 shows how the silver carp protein sequence can give rise to a variety of peptide fragments when subjected to the simulation of enzymatic hydrolysis with various proteolytic enzymes.

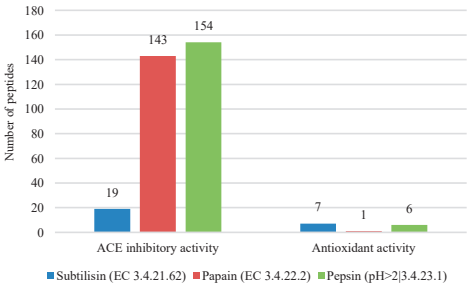

Figure 2. Prediction of the number of potential bioactive peptides in the *in silico* interaction of type I alpha-1 collagen with proteolytic enzymes

Of the three proteases selected for hydrolysis simulation, pepsin generated the highest number of bioactive peptides in the case of inhibitory ACE activity, namely 154. Papain showed 143 bioactive peptides, followed by subtilisin with 19 peptides with antihypertensive activity, generated from the chosen collagen protein.

The results for ACE inhibitory activity are in agreement with the data obtained by Zhang and co-workers (Zhang et al., 2019). They found pepsin to be able to hydrolyze a large number of peptide bonds from collagen substrate, generating ACE inhibitory activity. It is speculated that neutrase, acting at neutral pH, instead of pepsin, acting at acidic pH, could be a better choice for the generation of peptides

with ACE inhibitory and dipeptidyl peptidase IV inhibitory activities (Zhang et al., 2019).
The antioxidant activity was low for all three proteolytic enzymes. Subtilisin showed the highest activity, with a number of 7 peptides released, followed by pepsin with 6 peptides and papain with 1 peptide released.
Pal and Sureh (2017) have reported a small number of antioxidant peptide sequences in the *in silico* simulation of type I alpha-1 collagen proteolysis from *Ctenopharyngodon idella*. The researchers found a number of five antioxidant peptides released when papain was used as a hydrolysis protease and three peptides when digestion was simulated with pepsin (Pal & Suresh, 2017).
It is important to note that the ACE inhibitory activity was predominant when using these three enzymes in our study.
When we simulated the hydrolysis of collagen with a mix of two enzymes, pepsin + papain, a number of 121 peptides released with antihypertensive activity was observed, compared to 8 peptides in the case of antioxidant activity (Figure 3).

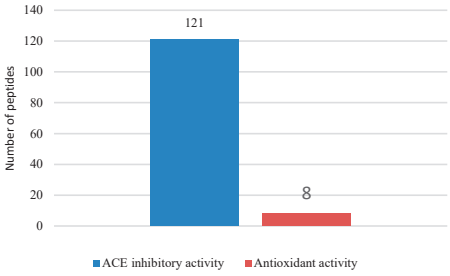

Figure 3. Prediction of the number of potential bioactive peptides in the *in silico* interaction of type I alpha-1 collagen with a mix of two enzymes (pepsin+papain)

In order to observe the potential of the peptides obtained from previous simulations, their profiles were subjected to bioactivity tests. For this purpose, the online tool PeptideRanker was used, which can classify peptides according to their bioactivity, based on structure-function analysis. Scores higher than 0.5 show a potential bioactivity (Pooja et al., 2017; Garg et al., 2018).
In the case of subtilisin, the most bioactive peptides with ACE inhibitory activity that are generated from type I alpha-1 collagen from silver carp (*H. molitrix*) were the MF (methionine – phenylalanine) peptide with a score of 0.99 and RF (arginine - phenylalanine) sequence with a score of 0.98 – detailed in Table 3.

Table 3. Bioactivity score of peptides* obtained from type I alpha-I collagen in the simulation with subtilisin

		Subtilisin		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	MF	0.996643	RHF	0.876772
2	RF	0.986556	VW	0.802223
3	PGL	0.855192	TY	0.113932
4	TF	0.826678	VY	0.0989681
5	GL	0.808777	-	-

*one letter amino acids abbreviation is used

The amino acid phenylalanine (F) was observed in several sequences. This amino acid was reported to generate bioactivity in short peptides (Mooney et al., 2012).
For the antioxidant activity, the RHF (arginine – histidine – phenylalanine) tripeptide showed a high score of 0.87, followed by the VW sequence with a score of 0.80. The TY and VY peptide sequences showed a bioactivity score below 0.5
The amino acid phenylalanine was also observed in the peptides obtained from papain (Table 4).

Table 4. Bioactivity score of peptides* obtained from type I alpha-I collagen in the simulation with papain

		Papain		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	SF	0.948796	IR	0.332363
2	NF	0.941145	-	-
3	PG	0.877086	-	-
4	MKG	0.559174	-	-
5	AG	0.546994	-	-

*one letter amino acids abbreviation is used

The SF (serine-phenylalanine) sequence was ranked 1st, with a bioactivity score of 0.948, followed by the NF (asparagine-phenylalanine) peptide with a score of 0.941 and PG peptide with a score of 0.87. The tripeptide MKG (methionine-lysine-glycine) was observed in the 4th place, with a score of 0.55. Significant antioxidant activity was not observed at the

hydrolysis simulation of collagen type I alpha-1 from silver carp (*H. molitrix*) with papain.
Pepsin showed high scores for WG, tryptophan-glycine (0.99), and SF (serine-phenylalanine) (0.94) sequences at ACE inhibitory activity (Table 5).

Table 5. Bioactivity score of peptides obtained from type I alpha-I collagen in the simulation with pepsin

		Pepsin		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	WG	0.992384	WG	0.992384
2	SF	0.948796	WY	0.974885
3	PG	0.877086	RHF	0.876772
4	RG	0.738353	VY	0.0989681
5	RL	0.626352	-	-

The WG (tryptophan - glycine) sequence was also observed in the case of antioxidant activity, followed by the WY (tryptophan - tyrosine) peptide with a score of 0.97 and the RHF (arginine- histidine – phenylalanine) tripeptide with a score of 0.87.
Table 6 shows the score of the peptides obtained from the combination of two enzymes: pepsin and papain.

Table 6. Bioactivity score of peptides obtained from type I alpha-I collagen in the simulation with a mix of two enzymes

		Pepsin+Papain		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	WG	0.992384	WG	0.992384
2	SF	0.948796	WY	0.974885
3	PG	0.877086	IR	0.332363
4	PR	0.787626	VY	0.0989681
5	IG	0.501816	-	-

In the case of ACE inhibitory activity, the first three peptides are similar to those obtained from pepsin. Additional 2 sequences, PR (proline-arginine) and IG (isoleucine-glycine), with scores of 0.78 and 0.50, respectively, were generated. The latter two peptides have also been reported by Ningrum and Munawaroh (2019). They used an *in silico* approach to observe the peptides released with antihypertensive activity when pepsin and papain together interact with type I collagen in the tuna fish. In the study of these researchers, PeptideRanker showed a score of 0.99 for the PR peptide and 0.54 for the IG peptide (Ningrum & Munawaroh, 2019). The different score in the case of PR peptide obtained in our study concerning that observed by Ningrum and Munawaroh is probably due to the update of the PeptideRanker program in the last years.
In the case of antioxidant activity, the WG (tryptophan – glycine) and WY (tryptophan – phenylalanine) sequences are the only peptides that showed significant bioactivity and are similar to those obtained from pepsin.
The most bioactive peptides obtained from the interaction of collagen with the three enzymes were subjected to the prediction of physico-chemical characteristics and the prediction of toxicity (Table 7).

Table 7. Prediction of toxicity and physicochemical characteristics of the most bioactive peptides obtained from silver carp collagen

Peptide	Molecular weight (g/mol)	Isoelectric point	Water solubility	Toxicity prediction	Taste
MF	296.39	3.45	low solubility	non-toxic	-
RF	321.37	10.55	good solubility	non-toxic	bitter
PGL	285.34	4.08	low solubility	non-toxic	-
TF	299.29	3.39	low solubility	non-toxic	-
GL	188.22	3.63	low solubility	non-toxic	bitter
RHF	458.51	10.55	good solubility	non-toxic	-
VW	303.63	3.57	low solubility	non-toxic	-
SF	252.27	3.43	low solubility	non-toxic	-
NF	279.29	3.28	low solubility	non-toxic	-
PG	172.18	4.06	low solubility	non-toxic	bitter
MKG	334.44	9.88	good solubility	non-toxic	-
AG	146.14	3.69	low solubility	non-toxic	-
WG	261.28	3.5	low solubility	non-toxic	-
RG	231.25	10.55	good solubility	non-toxic	bitter/ salt enhancer
RL	287.36	10.55	good solubility	non-toxic	bitter
WY	367.4	3.51	low solubility	non-toxic	-
PR	271.32	11.29	good solubility	non-toxic	bitter
IG	188.22	3.63	low solubility	non-toxic	bitter

Most of the peptides generated have a low molecular weight of 0.1-0.4 kDa. The peptide with the highest molecular weight (0.458 kDa) is RHF, followed by the peptides: WY (0.367

kDa), MKG (0.334 kDa), and RF (0.321 kDa). Most peptides showed an isoelectric point at acidic pH around 3 and were predicted to have low solubility in water. The peptides RF, RHF, MKG, RG, RL, and PR, showed the isoelectric point at alkaline pH, between 9 and 11, and were predicted to be soluble in water. The peptides analyzed do not contain amino acid residues considered potentially toxic, ToxinPred classifies all peptide sequences as non-toxic. It has been demonstrated that amino acids such as valine, threonine, arginine, glutamine, methionine, leucine, lysine, isoleucine, phenylalanine, or alanine are non-toxic, alone or in peptide sequences. Amino acids such as proline, histidine, cysteine, and asparagine have been reported to induce toxicity when they are included in some specific biotoxic peptide sequences (Ningrum & Munawaroh, 2019).

In conclusion, no toxic peptides were found for the threshold value set to 0 of the support vector machine, which makes these peptides good targets for further study.

Moreover, most of the peptides analyzed with ACE inhibitory activity showed bitter sensory characteristics. In addition to the bitter taste, the RG (arginine-glycine) peptide also showed salty sensory characteristics. Antioxidant peptides did not show taste characteristics (Table 7).

The last objective of this study was to predict the number of peptides with sensory characteristics resulting from the interaction of the three enzymes with type I alpha-1 collagen from silver carp (*H. molitrix*). Taste is an important indicator in evaluating food, which can help identify possible toxic substances. Peptides generated from various protein structures can affect food taste due to the presence of specific amino acids (Maehashi & Huang, 2009; Ningrum & Munawaroh, 2019).

Using the BIOPEP-UWM database, we found most of the peptides having bitter and umami tastes. We observed that pepsin generated a number of 198 bitter peptides, followed by papain with 87 peptides and subtilisin with 10 bitter peptides. The number of umami peptides was significantly smaller. Papain generated 18 umami peptides, pepsin 17 peptide sequence with umami taste and subtilisin one umami peptide (Figure 4).

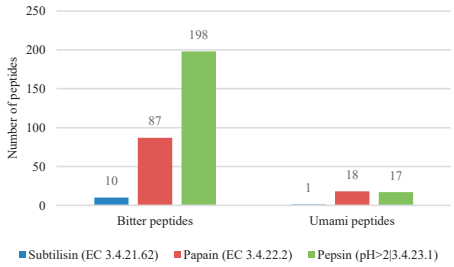

Figure 4. Prediction of the number of bitter and umami peptides in the *in silico* interaction of type I alpha-1 collagen from silver carp (*H. molitrix*) with different enzymes

CONCLUSIONS

This study is the first to show the advantages of using an *in silico* approach to obtain bioactive peptides from type I alpha-1 collagen in silver carp (*Hypophthalmichthys molitrix*).

The analysis of the profile of the potential biological activity of collagen showed several types of possible bioactivities, including antihypertensive and antioxidant activity.

Several non-toxic peptide sequences with possible beneficial bioactivity and appropriate physicochemical properties have been identified and can be utilized for more in-depth studies.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Research, Innovation and Digitization, CCCDI – UEFISCDI, project number PN-III-P2-2.1-PTE-2019-018, contract 3PTE-COL-STIM, within PNCDI III. We also thank the Ministry of Research, Innovation and Digitization, Program 1 - Development of the national research and development system, Subprogram 1.2 - Institutional performance - Projects to finance excellence in RDI, Contract no. 31PFE / 2018 and Contract no 15PFE / 2021, for financial support.

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