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BIOSYNTHESIS OF SEROTONIN IN BACTERIA: AN EXPLORATORY STUDY

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Abstract

Serotonin is a phylogenetically ancient compound found in all animals, plants, and some prokaryotes. In eukaryotes, serotonin is synthetized from the aromatic amino acid tryptophan via two key enzymes – Aromatic Amino Acid Hydroxylase (AAAH) and Aromatic Amino Acid Decarboxylase (AAAD). Serotonin is also an intermediate in the melatonin biosynthetic pathway and is generally involved in several vital functions.

In humans, serotonin is produced in the gut and in the brain and is critical in the regulation of multiple body functions, such as mood, cognition, appetite, and immunity. Serotonin depletion has been implicated in both neurological disorders, such as depression and Alzheimer's, as well as multiple peripheral conditions, such as IBS and fibromyalgia.

While the serotonin biosynthetic pathway is well described in eukaryotes, very little is known about how it occurs in bacteria. Evidence points to similar pathways as the ones described for eukaryotes, as eukaryote-like AAAH and AAAD have been found in multiple bacteria, even though serotonin production has not yet been detected in most species. Although no bacterial tryptophan hydroxylase genes have been described, evidence seems to hint that serotonin production in bacteria might occur through different AAAH and AAAD, as substrate promiscuity has been effectively reported in these enzymes before.

Serotonin biosynthesis has been previously detected in members of the genus *Pseudomonas*, and both AAAH and AAAD genes have been identified in *Pseudomonas putida*. The aim of this study was to test the substrate specificity of a putative AAAH from *P. putida*, previously annotated as phenylalanine hydroxylase. Enzymatic activity tests using different aromatic substrates under different biochemical conditions were performed. However, in the tested conditions, AAAH activity was only detected in the presence of phenylalanine, but not with tyrosine, tryptamine, or tryptophan. Even though the properties of *P. putida* AAAH determined in this study could not confirm what was initially hypothesized, this is still, for sure, a very promising field of research.

Considering the human gut microbiota as a potential source of serotonin, further investigation on these biosynthetic pathways in microbes might lead to important discoveries, which may then be translated into human conditions and, ultimately, to the development of new therapeutic strategies to treat serotonin depletion related disorders.

Keywords: serotonin biosynthesis; *Pseudomonas putida*; aromatic amino acid hydroxylase; substrate promiscuity; gut microbiome.

Resumo

A serotonina é um composto filogeneticamente antigo presente em todos os animais, plantas e em muitos organismos procariotas. Em eucariotas, a serotonina é sintetizada a partir do aminoácido aromático triptofano através de duas enzimas chave - Hidroxilase de Aminoácidos Aromáticos (AAAH) e Descarboxílase de Aminoácidos Aromáticos (AAAD). A serotonina é também um intermediário na via de síntese da melatonina. Em humanos, a serotonina é produzida no intestino e no cérebro, sendo essencial na regulação de múltiplas funções vitais, como humor, cognição, apetite e imunidade. A depleção de serotonina tem sido implicada em vários distúrbios neurológicos, como a depressão e a Doença de Alzheimer, e em várias condições periféricas, como a IBS e a fibromialgia.

Embora a via biossintética da serotonina esteja bem descrita em eucariotas, sabe-se ainda muito pouco sobre como esta ocorre em bactérias. Evidências apontam para vias semelhantes às descritas para eucariotas, uma vez que AAAH e AAAD semelhantes às de eucariotas foram já identificadas em várias bactérias, embora a produção de serotonina ainda não tenha sido efetivamente detetada na maioria destas espécies. Embora os genes para hidroxilases de triptofano não tenham ainda sido descritos em bactérias, evidências parecem sugerir que a produção de serotonina em bactérias possa ocorrer através de AAAH e AAAD diferentes, já que a promiscuidade de substrato foi efetivamente descrita para essas enzimas.

A biossíntese de serotonina foi previamente detetada em membros do género *Pseudomonas*, e genes de AAAH e AAAD foram identificados em *Pseudomonas putida*. O objetivo deste estudo foi testar a especificidade do substrato de uma AAAH de *P. putida*, previamente anotada como hidroxilase de fenilalanina. Testes de atividade enzimática utilizando diferentes substratos aromáticos sob diferentes condições bioquímicas, foram efetuados. No entanto, a atividade da AAAH só foi detetada na presença de fenilalanina, não sendo observada atividade com tirosina, triptamina ou triptofano. Ainda que propriedades da AAAH de *P. putida* determinadas neste estudo não confirmem a hipótese inicial, esta é certamente uma área de investigação muito promissora. Considerando o microbioma intestinal humano como uma potencial fonte de serotonina, a investigação destas vias biossintéticas em micróbios pode levar a descobertas importantes que poderão ser traduzidas para o sistema humano e, eventualmente, possibilitar o desenvolvimento de estratégias terapêuticas inovadoras para distúrbios associados à deficiência de serotonina.

Palavras-chave: biossíntese de serotonina; *Pseudomonas putida*; hidroxilase de aminoácidos aromáticos; promiscuidade de substrato; microbioma intestinal.

List of Abbreviations

5-HT – 5-Hydroxytryptamine (serotonin)

5-HTP – 5-Hydroxytryptophan

AAA – aromatic amino acid

AAAD – aromatic amino acid decarboxylase

AAAH – aromatic amino acid hydroxylase

ADHD - attention deficit hyperactivity disorder

AnPRT - anthranilate phosphoribosyltransferase

ASD – autism spectrum disorder

ASMT - N-acetylserotonin-O-methyltransferase

BA – biogenic amine

BBB – blood-brain barrier

BH_4 – tetrahydrobiopterin

BP – bipolar disorder

CBD - chitin-binding domain

CD – Crohn's disease

CNS – central nervous system

CS – chorismate synthase

DAHPS – deoxy-D-arabino-heptulosonate-phosphate synthase

DHQS – dehydroquinate synthase

DQD – dehydroquinate dehydratase

EC – enterochromaffin cell

ENS – enteric nervous system

EPSPS – enolpyruvylshikimate phosphate synthase

GMQL - global model quality estimate

HPLC-MS – high performance liquid chromatography mass spectrometry

IBD – irritable bowel syndrome

IBS – inflammatory bowel syndrome

IFE – inner filter effect

IGP – indole-glycerol-phosphate

L-DOPA - L-dihydroxyphenylalanine

mRNA – messenger ribonucleic acid

NAD^+ - nicotinamide adenine dinucleotide

$NADP^+$ - nicotinamide adenine dinucleotide phosphate

$NADPH_2$ - nicotinamide-adenine dinucleotide phosphate (reduced)

OCD – obsessive compulsive disorder

Phe – phenylalanine

PheH – phenylalanine hydroxylase

PLP - pyridoxal phosphate

PRAI – phosphoribosyl anthranilate isomerase

PTSD – post-traumatic stress disorder

RFU - relative fluorescence units

ROS – reactive oxygen species

SCFA – short-chain fatty acids

SDS-PAGE - sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SK – shikimate kinase

SKDH – shikimate dehydrogenase

SNAT – serotonin-N-acetyltransferase

TLC – thin layer chromatography

Trp – tryptophan

TrpAB - tryptophan synthase

TrpH – tryptophan hydroxylase

Try – tryptamine

Tyr – tyrosine

TyrH – tyrosine hydroxylase

UC – ulcerative colitis

Chapter 1 - Introduction

1.1. Aromatic amino acids

Amino acids are small molecules that include amine (NH_2) and carboxyl (COOH) functional groups in their biochemical structure and are crucial in both eukaryotic and prokaryotic biological systems. To date, 22 amino acids are known to be involved in protein assembling, from which 20 of them are found in the genetic code and are identified as proteinogenic amino acids. Among these 20 proteinogenic amino acids, there are 9 essential amino acids for humans, which must be obtained exogenously since the body cannot synthetize them, and 11 non-essential amino acids, synthetized from essential amino acids (Table 1). Two other amino acids found in proteins, selenocysteine and pyrrolysine, can only be incorporated into proteins through special translational mechanisms. Pyrrolysine is not known to occur in human proteins, while selenocysteine is found in proteins of the three domains of life, albeit at a very low rate (Ayon, 2020).

Tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr) are the three classic aromatic amino acids (AAA). These compounds are included in this group because they contain an aromatic ring in their chemical structure. Phenylalanine is aromatic due to its phenyl group, tyrosine for its 4-hydroxy-phenyl group and tryptophan because of its heterocyclic indole ring. AAA are biologically vital, as they play crucial roles in protein structural and catalytic functions and are also involved in the biosynthesis of multiple essential secondary metabolites (Parthasarathy et al., 2018).

Table 1. List of essential and non-essential proteinogenic amino acids (adapted from Ayon, 2020).

Essential amino acids	Non-essential amino acids
Histidine (His)	Alanine (Ala)
Isoleucine (Iso)	Arginine (Arg)
Leucine (Leu)	Asparagine (Asn)
Lysine (Lys)	Aspartic acid (Asp)
Methionine (Met)	Cysteine (Cys)
Phenylalanine (Phe)	Glutamine (Gln)
Threonine (Thr)	Glutamic acid (Glu)
Tryptophan (Trp)	Glycine (Gly)
Valine (Val)	Proline (Pro)
	Serine (Ser)
	Tyrosine (Tyr)

1.1.1. Aromatic amino acid metabolism

Plants, algae, bacteria, fungi and even some protozoa are capable of synthetizing AAA through the shikimate pathway, illustrated in Figure 1. The general enzymatic reactions and the metabolic intermediaries involved in this pathway seem to be shared in all organisms where this pathway is present, although the specific structure and subcellular location of the enzymes may vary. The shikimate pathway is similar for all three AAA biosynthetic pathways up until the chorismate synthesis step, from which it differs depending on the final AAA and the organism producing them (Tzin, Galili, & Aharoni, 2012). The AAA biosynthetic pathways known to date are illustrated in Figure 2. Biosynthesis of phenylalanine and tyrosine both start with the conversion of chorismate into prephenate, by chorismate mutase, both in plants and in microorganisms. From there, the pathways diverge. In bacteria, the bifunctional enzyme chorismate mutase/prephenate dehydratase can convert chorismate into prephenate, as well as prephenate into phenylpyruvate (for phenylalanine biosynthesis) or into 4-hydroxyphenylpyruvate (for tyrosine biosynthesis). The reactions in which phenylpyruvate and 4-hydroxyphenylpyruvate produce phenylalanine and tyrosine, respectively, are both catalyzed by AAA aminotransferase. In plants, arogenate is produced from prephenate, by prephenate aminotransferase. Arogenate may then be converted into tyrosine, by arogenate dehydrogenase, or into phenylalanine, by arogenate dehydratase. Tryptophan biosynthesis from chorismate occurs in a 6-step reaction that starts with chorismate conversion into anthranilate, by Anthranilate Synthase (AS). This enzyme is allosterically inhibited by tryptophan and its structure differs between organisms. Anthranilate later originates phosphoribosylanthranilate through Anthranilate Phosphoribosyltransferase (AnPRT) activity. Phosphoribosyl Anthranilate Isomerase (PRAI) then catalyzes the isomerization of N-(5-phospho-beta-D-ribosyl) anthranilate into 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate, which is then converted into indole-3-glycerol-phosphate (IGP), by Indole Glycerol Phosphate Synthase (IGP Synthase). The last two steps of the reaction are catalyzed by Tryptophan Synthase (TrpAB). This enzyme is an $\alpha_2\beta_2$ tetramer and has two separate active sites that are connected to each other and allow the enzyme to catalyze two different reactions. The α subunit cleaves IGP into indole and glyceraldehyde-3-phosphate, and then directs its products into the second active site, in the β subunit, where tryptophan is finally generated (Parthasarathy et al., 2018).

The shikimate pathway and the AAA biosynthesis pathways are often under post-translational regulation, through allosteric feedback loops (in bacteria), and/or transcriptional regulation, through transcription factors (in plants and some yeasts) (Tzin, Galili, & Aharoni,

2012). These amino acids are then used as building blocks in the synthesis of multiple other important specialized metabolites, such as auxins, indole alkaloids, glucosinolates, phenylpropanoids and plastoquinones (Parthasarathy et al., 2018).

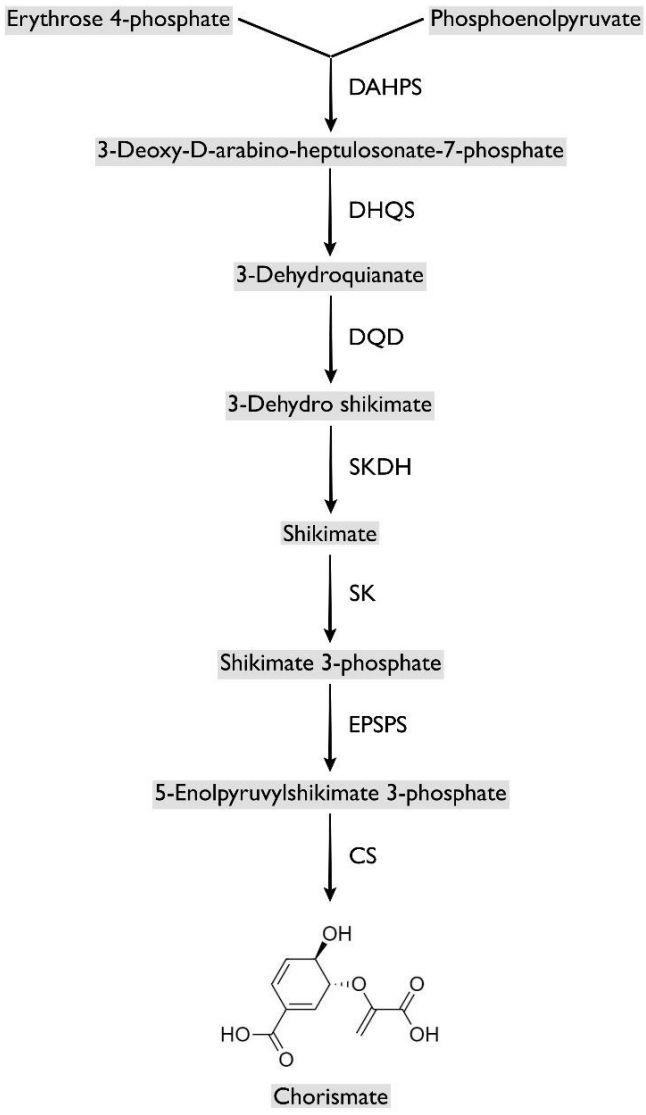

Figure 1. Scheme of the shikimate pathway. The illustrated reaction converts phosphoenolpyruvate and erythrose 4-phosphate into chorismate through a 7-step reaction catalyzed by multiple enzymes: DAHPS = 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase; DHQS = 3-Dehydroquinate Synthase; DQD = 3-Dehydroquinate dehydratase; SKDH = Shikimate 5-dehydrogenase; SK = Shikimate Kinase; EPSPS = 5-Enolpyruvylshikimate 3-phosphate Synthase; CS = Chorismate Synthase (adapted from Tzin, Galili, & Aharoni, 2012).

Contrarily to plants and microbes, animals must obtain AAA through their diet as the shikimate pathway is not present in these organisms. After AAA uptake, these molecules can either go through complete degradation or be used as precursors in various vital pathways,

such as protein synthesis and biosynthesis of neurotransmitters and neurohormones. Phenylalanine can be converted into tyrosine and, ultimately, tyramine, L-DOPA, dopamine, epinephrine (adrenaline) and norepinephrine. Tryptophan is the primary precursor for serotonin, tryptamine, and melatonin, and also takes part in the synthesis of other compounds such as the vitamin niacin, cofactors NAD^+ and $NADP^+$, and neuroprotectant kynurenine.

As most of these metabolic processes are vital, AAA catabolism related deficiencies have serious health impacts and may lead to serious disorders (Parthasarathy et al., 2018).

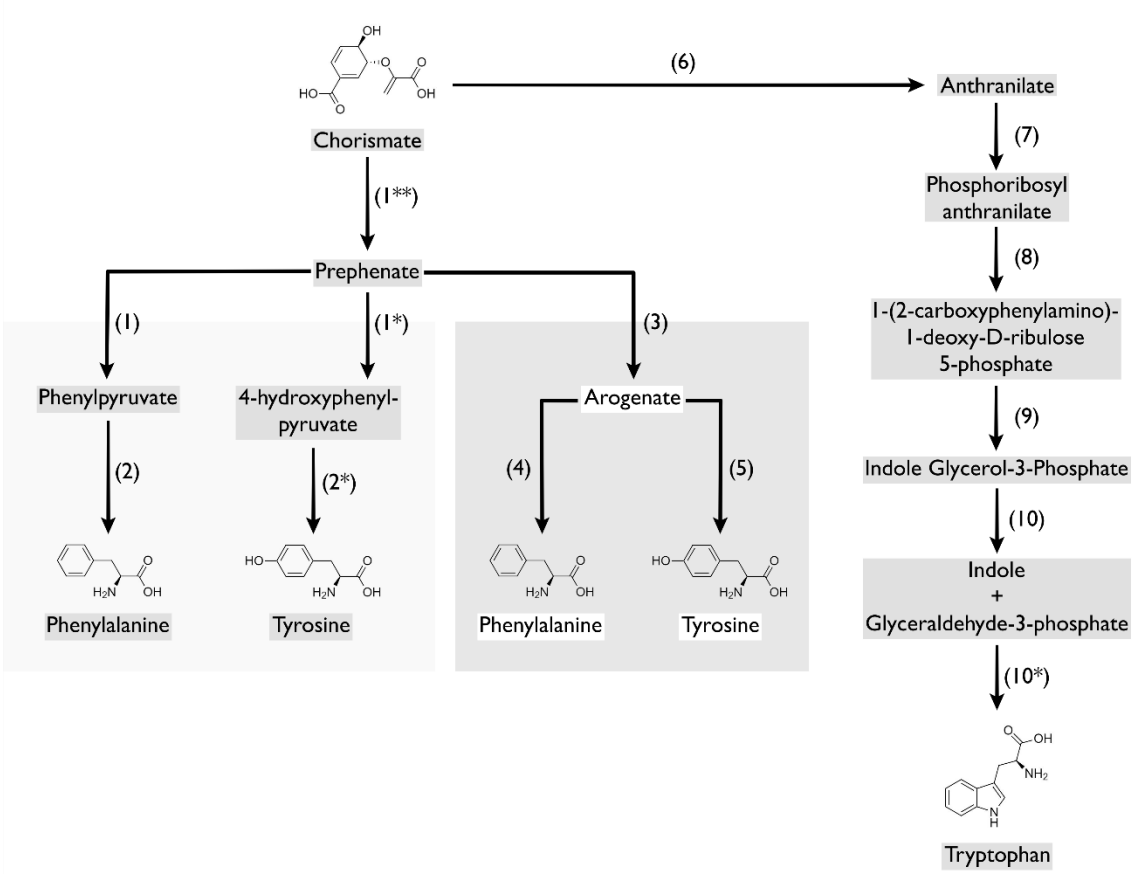

Figure 2. Aromatic amino acids biosynthetic pathways. After the chorismate synthetic step in the shikimate pathway, tryptophan, tyrosine and phenylalanine can be synthetized through different pathways. Phe and Tyr synthesis share the first step in which chorismate is converted into prephenate, by chorismate mutase/prephenate dehydratase (1**). In bacteria, the bifunctional enzyme chorismate mutase/prephenate dehydratase catalyzes the formation of both phenylpyruvate (1) and 4-hydroxyphenylpyruvate (1*) from prephenate. AAA aminotransferase then converts the latter two compounds into Phe (2) and Tyr (2*), respectively. In plants, Phe and Tyr synthesis occurs through conversion of prephenate into arogenate, by prephenate aminotransferase (3), and conversion of arogenate into Phe, by arogenate dehydratase (4), or into Tyr, by arogenate dehydrogenase (5). Tryptophan synthesis from chorismate occurs through a 6-step reaction catalyzed by anthranilate synthase (6), anthranilate phosphoribosyl synthase (7), phosphoribosyl anthranilate isomerase (8), indole glycerol-4-phosphate (9), tryptophan synthase (α subunit) (10) and tryptophan synthase (β subunit) (10*) (adapted from Parthasarathy, et al., 2018).

1.1.2. Aromatic amino acid hydroxylases (AAAH)

Aromatic amino acid hydroxylases (AAAH) are non-heme ferrous iron and tetrahydrobiopterin dependent monooxygenases that use oxygen to hydroxylate their substrates. They include tryptophan hydroxylase (TrpH), phenylalanine hydroxylase (PheH) and tyrosine hydroxylase (TyrH). These three enzymes catalyze three general vital reactions, illustrated in Figure 3.

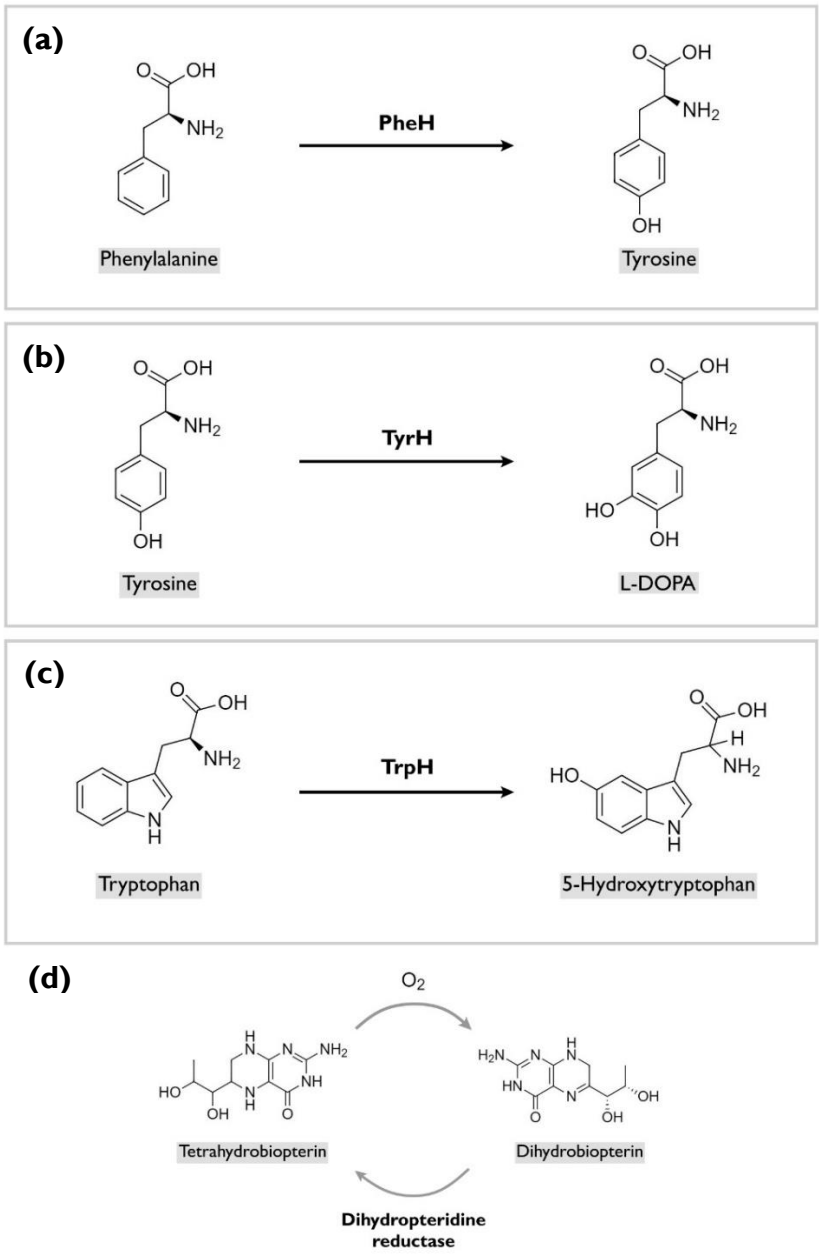

Figure 3. Reactions catalyzed by aromatic amino acid hydroxylases. (a) Phenylalanine hydroxylase (PheH) converts phenylalanine into tyrosine. **(b)** Tyrosine hydroxylase (TyrH) converts tyrosine into L-DOPA. **(c)** Tryptophan hydroxylase (TrpH) converts tryptophan into 5-hydroxytryptophan. These three reactions need tetrahydrobiopterin (BH_4), which is oxidized during the reaction, resulting in dihydrobiopterin formation, and BH_4 regeneration by dihydropteridine reductase, as shown in **(d)**.

AAAH are activated by iron in its ferrous form and require tetrahydrobiopterin (BH_4) as a cofactor, which reduces the second oxygen atom into water (Fitzpatrick, 2003) and is oxidized into dihydrobiopterin. Dihydrobiopterin is later regenerated back into BH_4 by the enzyme dihydropteridine reductase (Hufton, Jennings, & Cotton, 1995). In mammals, TrpH is present in the brain and in the gut, where it converts L-tryptophan into 5-hydroxytrytophan, the first step in the serotonin and melatonin biosynthetic pathway. PheH converts L-phenylalanine into L-tyrosine in the liver. L-tyrosine is hydroxylated into L-DOPA by TyrH in the central nervous system (CNS) and adrenal gland, as the first step in the synthesis of dopamine, norepinephrine and epinephrine, the three primary catecholamines.

Regarding their structure, the eukaryotic AAAH are homotetramers with three characteristic domains - the regulatory domain in the N-terminus; the catalytic domain near the C-terminus, responsible for substrate specificity; and the tetramerization domain, in the C-terminus (Fitzpatrick, 2003). Whereas the catalytic domain seems to be highly conserved and homologous between different AAAH, the presence of non-homologous N-terminal regulatory domains seems to influence the conformation and flexibility of the active site (Fitzpatrick, 1999).

1.1.3. Aromatic amino acid decarboxylases (AAAD)

Aromatic amino acid decarboxylases (AAAD) are pyridoxal 5'-phosphate (PLP) dependent enzymes that convert aromatic amino acids into amines (Koyanagi et al., 2012). AAAD catalyze multiple reactions including decarboxylation of L-DOPA into dopamine, 5-hydroxytryptophan (5-HTP) into serotonin, tryptophan into tryptamine, phenylalanine into phenylethylamine, tyrosine into tyramine, histidine into histamine, among others (Wishart et al., 2018). From what is known today, in eucaryotes, these genes seem to be frequently organized in clusters with other enzyme-encoding genes, usually synthetases, permeases and ion antiporters (Barbieri et al., 2019). This clustered structure has been identified in some bacterial AAAD genes as well (Mohedano et al., 2015).

1.2. Serotonin and melatonin chemistry and origins

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a multi-functional indolamine (Cox & Lee, 2016) whose structure contains an indole ring that can capture light, inherited from its precursor tryptophan. This function played a key role in primitive anaerobic unicellular organisms, allowing them to convert solar energy into biochemical energy, through carboxylase action.

Oxygen production, consequence of anaerobic metabolism, resulted in the accumulation of reactive oxygen species (ROS) inside the cells, which caused carboxylases to acquire their hydroxylase function as a mechanism of protection against the reactive oxidizing agents. These primitive hydroxylases used tryptophan, tyrosine and phenylalanine as substrates, binding oxygen to them and forming different antioxidant compounds, such as serotonin. Thus, serotonin is a phylogenetically ancient compound, and its biosynthesis has been conserved throughout evolution in different phyla, from primitive forms of life, like unicellular organisms, sponges and hydras, to the most complex ones, like vertebrates and plants (Yabut et al., 2019).

Serotonin is also an intermediate in the melatonin biosynthesis pathway. Melatonin (*N*-acetyl-5-methoxytryptamine) is a methoxyindole that was first isolated from the bovine pineal gland in 1958. Since then, this small molecule has been identified in various other animal tissues and in almost all living organisms including plants, fungi and bacteria (Tan et al., 2016). Just like serotonin, melatonin is a very ancient molecule that was already present in primitive organisms, such as cyanobacteria and α-proteobacteria, in which it had vital antioxidant and free radical scavenger functions (Zhao et al., 2019).

The principle that serotonin and melatonin biosynthesis was conserved throughout evolution can be explained by the endosymbiotic theory – cyanobacteria and α-proteobacteria were phagocytized by primitive eukaryotes, eventually establishing a symbiotic association with the host, and evolving into chloroplasts and mitochondria, respectively. Ultimately, divergent evolution caused serotonin and melatonin functions, biosynthetic pathways, generation sites and regulation to diverge between organisms (Zhao et al., 2019).

Although serotonin was first identified about 70 years ago because of its constricting effects on smooth muscle (Hensler, 2012), today it is known to take part in most central and peripheral functions in the human body. In the CNS, serotonin acts as a neurotransmitter involved in the regulation of multiple brain functions including sleep, mood, cognition, memory and sexual behavior. Peripherally, serotonin is associated to virtually all major organ systems and has a critical role in energy balance, appetite, gut motility, immunity, liver repair (Yabut et al., 2019) and cardiovascular and pulmonary physiology, among others (Berger, Gray, & Roth, 2009).

Melatonin physiologically works as an endogenous synchronizer that stabilizes circadian rhythms and therefore regulates, either directly or indirectly, multiple body functions and mechanisms that depend on that periodicity, such as body temperature, induction of sleep, blood pressure, immune responses, cardiovascular regulation and antioxidant protection (Claustrat & Leston, 2015).

1.3. Serotonin and melatonin in animals

1.3.1. Biosynthesis and function

In humans and other animals, serotonin is synthetized from the amino acid L-tryptophan in a two-step reaction. L-tryptophan is initially hydroxylated by TrpH to form 5-HTP, which is then decarboxylated by an AAAD to produce serotonin (Yabut et al., 2019). Serotonin may then be used to synthetize melatonin, by being first acetylated into *N*-acetylserotonin by serotonin-*N*-acetyltransferase (SNAT), and then into melatonin, by *N*-acetylserotonin-*O*-methyltransferase (ASMT) (Pelagio-Flores & López-Bucio, 2016). These reactions are illustrated in Figure 4. These biosynthetic pathways are limited by tryptophan availability and by the rate limiting enzymes, TrpH and SNAT. Tryptophan hydroxylase requires tetrahydrobiopterin, oxygen, $NADPH_2$ and a metal to be catalytically active (Athar, 2010). SNAT's mRNA expression in the pineal gland is under the influence of day/night cycles, causing this enzyme to only be active in specific periods of time and conditions (Claustrat & Leston, 2015).

Serotonin is produced in very low quantities in animals, about 5-10 mg of serotonin in an average adult human, mainly due to tryptophan limitation, as this amino acid can only be obtained through diet and a large percentage of it is catabolized via the kynurenine pathway (Yabut et al., 2019). Over 90% of total human body serotonin is generated and located in the gut (Martin et al., 2019), with the remaining percentage being produced in the brain and other additional peripheral sites, such as pancreatic β-cells, osteoclasts and adipocytes. Central serotonin and peripheral serotonin are often considered two separate entities as these molecules are synthetized by two distinct TrpH isoforms (TrpH 1 in non-neuronal cells and TrpH 2 in neuronal cells) and serotonin cannot cross the blood-brain barrier (Spohn & Mawe, 2017).

In the gut, enterochromaffin cells (EC) synthetize serotonin using TrpH 1, whose activity and expression appears to be regulated by nearby cells and certain nutrients, such as carbohydrates (Yabut et al., 2019). Serotonin is then released in a mechanical and chemical stimulus-regulated way (Banskota, Ghia, & Khan, 2018) and takes part in multiple reactions, not only in the intestine, but also systemically by binding to circulating platelets in the bloodstream (Yabut et al., 2019).

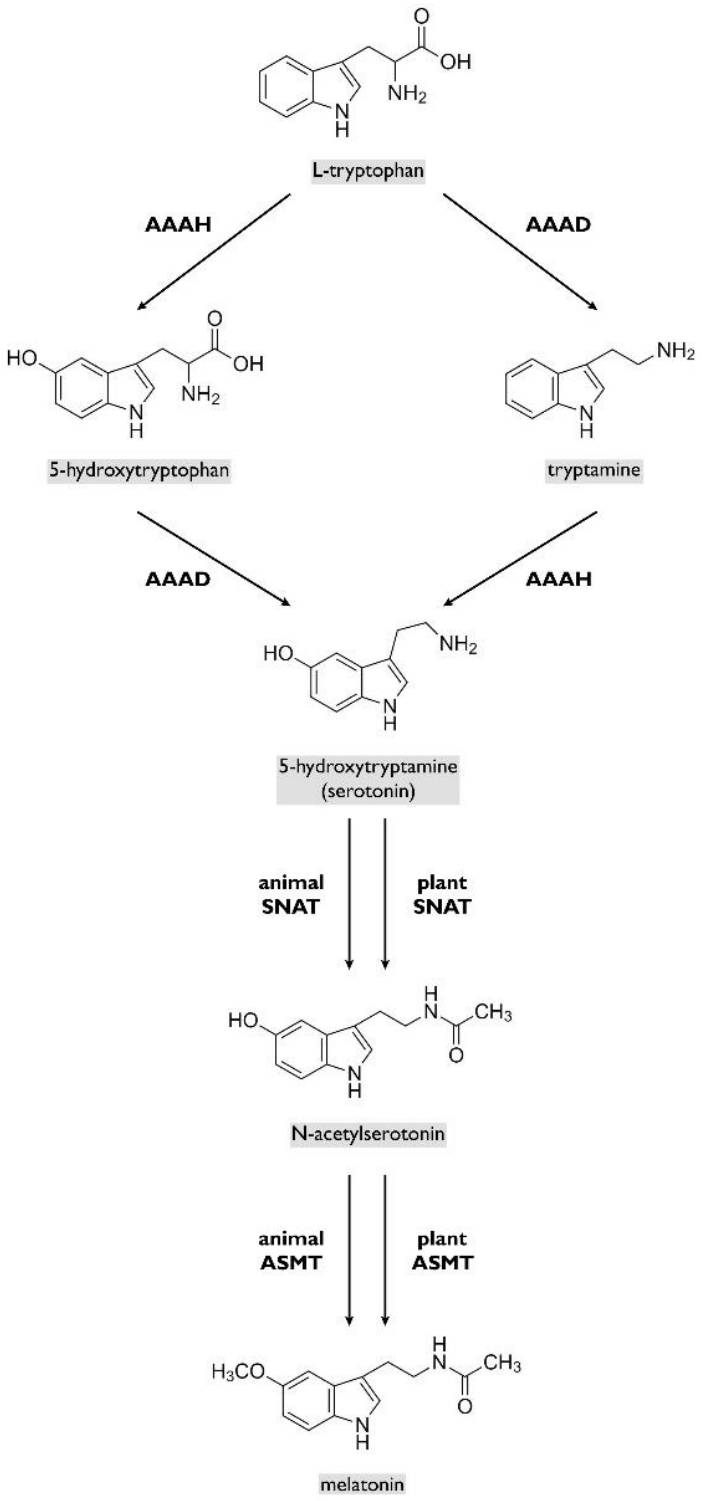

Figure 4. Serotonin and melatonin biosynthetic pathways in vertebrates (left side) and plants (right side). L-tryptophan is hydroxylated by tryptophan hydroxylase (TrpH) to form 5-hydroxytryptophan, which is then decarboxylated by an aromatic amino acid decarboxylase (AAAD) to produce serotonin. Serotonin is then acetylated into *N*-acetylserotonin by serotonin-*N*-acetyltransferase (SNAT), and finally into melatonin, by *N*-acetylserotonin *O*-methyltransferase (ASMT).

In the CNS, serotonergic neurons produce serotonin via TrpH 2 activity. This TrpH isoform is also used by enteric neurons in the enteric nervous system (ENS) (Bader, 2019).

Serotonin synthesis in the brain is highly limited by the quantity of tryptophan that crosses the brain-blood barrier as only free/unbound plasma tryptophan can do so (Athar, 2010). After synthesis, serotonin is encapsulated in vesicles and released by exocytosis into the neuronal synaptic cleft, activates specific receptors and is then removed from the synapse by presynaptic neurons reuptake (Gresch, 2013). This way, serotonin can be sent from the Raphe nuclei in the brainstem, where serotonergic neurons originate, to nearly all areas of the CNS (Bader, 2019).

As previously stated, melatonin secretion in humans is regulated by light/dark cycles. The photic information is transmitted through the retinohypothalamic tract to the suprachiasmatic nuclei of the hypothalamus, which in turn communicates with multiple areas in the brain, including the pineal gland, where melatonin is synthetized. However, melatonin synthesis is not exclusive to the pineal gland. Studies estimate that about 99% of total body melatonin is produced in other tissues, although it is never released into the bloodstream and therefore its circulating quantities are not systemically significant. In the presence of light, during the day, melatonin synthesis in the pineal gland is inhibited; its levels peak at night, when it is released into circulation, reaching all body tissues, including the brain as it is able to cross the blood-brain barrier (BBB), and because melatonin is highly soluble in lipid and water, it can easily cross cell membranes (Claustrat & Leston, 2015). Melatonin receptors are widely distributed throughout the human body and, even though some effects of melatonin cannot be explained by membrane receptors or by ROS scavenging, many of its activities known to date are mediated by transmembrane G-protein coupled receptors, which activate signaling cascades in the target cells (Zhao et al., 2019).

1.3.2. Serotonin deficiency disorders

As previously mentioned, the serotonergic system is involved in multiple CNS functions and its homeostasis is crucial to maintain proper brain function (Athar, 2010). Serotonergic neurons are also responsible for controlling ENS development, influencing neurogenesis and ensuring the survival of the new developing neurons. Although serotonin has a critical role in the CNS and ENS function, the highest percentage of serotonin is in the gut enterochromaffin cells (O'Mahony et al., 2014).

Dysregulation of serotonergic signaling pathways has been previously implicated in many psychiatric and neurological conditions such as pain, sleep problems, appetite and mood variations (Athar, 2010), and disorders like depression, anxiety, schizophrenia, attention deficit hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD), migraine headache,

autism spectrum disorders (ASD) (Pourhamzeh et al., 2021), bipolar disorder (BP) and addictive behavior (Lin et al., 2014). Serotonin depletion has also been implicated in neurodegenerative diseases, such as Alzheimer's Disease, as serotonin levels decrease with age and neuroinflammation processes have been associated with reduction of serotonin synthesis' precursors in the CNS (Danilovich, Alberto, & Juárez Tomás, 2021). Additionally, lower levels of serotonin have been detected in fibromyalgia patients, where this imbalance has also been associated with other key symptoms of this pathology, such as fatigue and cognitive deficits (Welsch et al., 2018). Outside the CNS, dysregulation of serotonin levels has been connected to gastrointestinal disorders. Studies report deviations in serotonin levels in inflammatory bowel disease (IBD) and colitis. Increased serotonin-producing EC cell quantity has been observed in Crohn's disease (CD) and in ulcerative colitis (UC) patients. These gut inflammatory conditions have been linked to serotonin-dependent angiogenesis, as serotonin has angiokine function in endothelial cells and increased vascularity has been observed in colitis and IBD. Disruption of serotonin balance levels has also been previously associated with irritable bowel syndrome (IBS), celiac disease, neuroendocrine tumors, and metabolic disorders, such as obesity and type 2 diabetes (Banskota, Ghia, & Khan, 2018). Melatonin level dysfunction is most associated with sleep disorders. However, as it controls multiple systemic circadian rhythm-dependent processes, its deregulation is also implicated in other conditions such as epilepsy, autism, anxiety, bipolar disorder, and depression (Danilovich, Alberto, & Juárez Tomás, 2021).

Due to their vital functions on the human body, serotonin and melatonin are therapeutically relevant in many disorders. Serotonergic system's components (such as serotonin receptors) are commonly used as pharmacological targets, which has proven to be very effective in the treatment of many neurological conditions, such as depression through selective serotonin reuptake inhibitor drugs. Melatonin is extensively used to treat sleep associated problems, such as insomnia and jetlag, through oral administration. Additionally, pioneering studies have been evidencing its beneficial effects on prevention and treatment of age-associated neurodegenerative diseases, like Alzheimer's, Parkinson, and Huntington, due to its anti-inflammatory and antioxidant properties. Clinical studies with cancer patients have also demonstrated that melatonin oral administration decreases the toxicity of chemotherapeutic drugs. Due to its cell protection and damage repair activity, melatonin has also shown to be effective in preventing cell tumorigenic mutations in animal models of cancer. Moreover, this indolamine has also been indicated as an effective coadjuvant therapy in gut diseases and in parasitic infections, due to its link with the immune system (Danilovich, Alberto, & Juárez Tomás, 2021).

1.4. Serotonin and melatonin in plants, fungi and unicellular organisms

Serotonin production in plants was first described in 1954 in *Mucuna pruriens* (Bowden, Brown, & Batty, 1954). Melatonin in plants was only reported later in 1993 by Van Tassel *et al.* in *Pharbitis nil* and *Solanum lycopersium*. Since then, these compounds have been identified in multiple other plant species where their levels are highly variable, depending on the species and on the plant tissue (Kang et al., 2007).

Phytoserotonin appears to be highly important throughout all plant life stages, including germination, growth, reproduction, and senescence, as well as stress survival and tolerance (Erland, Turi, & Saxena, 2019). Its average content is estimated to be 100x higher than that in the animal brain (Azmitia, 2007) and its levels can go up to 400 µg/g in walnuts (*Juglans regia*) (Pelagio-Flores & López-Bucio, 2016).

Phytomelatonin is also highly involved in the regulation of multiple plant life processes such as seed protection, germination, vegetative growth, root development, fruit maturation and senescence delay (Zhao et al., 2019) and has previously been associated with circadian rhythm regulation (Pelagio-Flores & López-Bucio, 2016). Alike phytoserotonin, phytomelatonin plays a very important role in stress tolerance as well (Zhao et al., 2019). Melatonin levels have been reported to go up to 230 µg/g in *Pistachio* kernels (Pelagio-Flores & López-Bucio, 2016).

Contrary to animals, plants retained the ability to synthetize tryptophan during evolution. That, along with the fact that plants have both mitochondria and chloroplasts, in both of which melatonin and serotonin are produced, seems to make the biosynthetic processes of these molecules more productive, when compared to animals. Evolution wise, these features were probably crucial since plants, as immobile beings, cannot behaviorally escape environmental threats. For that reason, they need to respond more quickly and more strongly to those stressful conditions to survive. Those stressful conditions boost ROS production and oxidative damage, triggering defense mechanisms such as antioxidant production. Multiple studies have shown that melatonin levels rise in response to various environmental insults (Zhao et al., 2019) and that serotonin levels are higher in plant tissues undergoing stress (Erland, Turi, & Saxena, 2019).

The serotonin and melatonin synthetic pathways in plants differ from those in humans and other vertebrates. While in vertebrates, serotonin biosynthesis occurs through L-tryptophan hydroxylation followed by decarboxylation, in plants the process is reversed – L-tryptophan is first decarboxylated into tryptamine, by tryptophan decarboxylase, followed by

hydroxylation of tryptamine into serotonin, by tryptamine-5-hydroxylase (Figure 4) (Kang et al., 2007). Serotonin may then be converted into *N*-acetylserotonin, by serotonin-*N*-acetyltransferase (SNAT), and then into melatonin, by *N*-acetylserotonin *O*-methyltransferase (ASMT). Although the conversion of serotonin into melatonin is similar in animals and plants, SNAT and ASMT are not homologous between these two groups (Erland, Turi, & Saxena, 2019). However, the biosynthetic pathway described before may not be universal to all plants as some studies reported possible alternative pathways, such as serotonin synthesis via 5-hydroxytryptophan in *Hypericum perforatum* (Murch, KrishnaRaj, & Saxena, 2000), and melatonin synthesis through serotonin methylation by caffeic acid-*O*-methyltransferase in *Arabidopsis thaliana* (Byeon et al., 2014).

Although it is known that serotonin and melatonin are used by virtually all organisms, there is little evidence on their production in organisms other than animals and plants, as the greater part of the research is focused on these two groups. Melatonin has been previously detected in wine and beer, which is thought to be linked to yeast metabolism, such as fermentation processes in *Saccharomyces* yeasts. Serotonin is also found in wines and its biosynthesis has been reported in yeasts exposed to UV radiation (Mas et al., 2014). The protozoan parasite *Entamoeba histolytica* secretes serotonin, which seems to be responsible for some of its infection symptoms in animals (McGowan et al., 1983). Serotonin biosynthesis in bacteria has also been reported occasionally and will be discussed in a further section.

1.5. The human gut microbiome

The gut microbiome comprehends the complex and dynamic microbial community present in the mammalian gastrointestinal tract (Galland, 2014) where it functions as a "metaorganism" that established a symbiotic and commensal relationship with the mammalian host (Hill, 2014). Human gut bacterial cells are estimated to be at a ratio of approximately 1:1 with our own cells and over 9 million genes have been identified so far (Thursby & Juge, 2017). The human gut microbial community holds thousands of different species from which about 99% are anaerobic bacteria, with the remaining percentage comprising fungi, viruses, protozoa, archaea and other microorganisms (Hill, 2014). Even though the human gut microbiota structure is highly specific for each individual, two main bacterial divisions classically dominate this community, Bacteroidetes and Firmicutes, and in smaller percentages, Proteobacteria, Fusobacteria, Tenericutes, Actinobacteria and Verrucomicrobia. These phyla are estimated to make up about 90% of the total human gut microbiota (Gomaa, 2020).

Typically, bacteria do not naturally exist in isolation, they tend to form clusters of communities that co-exist and interact. These clusters in the human gut microbiome are often referred to as enterotypes and although the factors that cause this clustering are not fully understood yet, these vary in bacterial species and functional structure across individuals. Further investigation might contribute to the development of new diagnostic, prognostic, and therapeutic strategies for multiple diseases (Arumugam, Raes, & Pelletier, 2011). Furthermore, while the analytical and biological parameters are not easy to establish, studies have been focusing in defining a core microbiota hypothetically shared across human adults (Salonen et al., 2012).

The human gut microbiota composition fluctuates across the lifespan. Although the microbial gut colonization is thought to be initiated only at birth, a few studies have reported detection of microbes in womb tissues. The diversity and stability of the human gut microbiota are limited in the first 2,5 years of life. From there and through childhood it develops and fairly stabilizes through adulthood. In later stages of life, the gut microbiota composition suffers alterations and inter-individual variability peaks. Through these life stages, the inter-individual gut microbiota composition variation seems to be determined by multiple intrinsic and external factors (Thursby & Juge, 2017). These factors may include host genetics, gestational age (preterm or healthy term), mode of birth (vaginal delivery or cesarian section), early nutrition (breast-fed or formula-fed), weaning and solid food introduction, type of diet, dietary pattern alterations, probiotic and prebiotic ingestion, antibiotic usage and even certain geographical and cultural factors (O'Mahony et al., 2014). These gut microbial community fluctuations are tolerated due to our GI tract composition, which evolved to create a complex and dynamic barrier of epithelial and mucus layers, enzymes, antimicrobial compounds, and immune system components, protecting us against damage and preserving our system homeostasis (Thursby & Juge, 2017).

The gut microbiome has been gathering more and more attention by the scientific community in the last decade as studies have been uncovering the vast impact it has on human health through mechanisms not yet fully elucidated. Its protecting, structural and metabolic functions, such as food processing, pathogen displacement and synthesis of vitamins, are not restricted to the gut and the ENS, as the microbial-produced and regulated compounds are released into the bloodstream, reaching and acting at other distal organs and tissues, exerting important endocrine effects as well. These microbiota-secreted compounds include short-chain fatty acids (SCFA), neurotransmitters and some of their precursors, hypothalamic-pituitary-adrenal hormones, gastrointestinal hormones, and various others.

Collaborative metagenomic-based studies like the Human Microbiome Project (http://commonfund.nih.gov/hmp), the Metagenomics of the Human Intestinal Tract Project (https://www.gutmicrobiotaforhealth.com/metahit) and the International Human Microbiome Consortium (http://www.human-microbiome.org) have been extremely important in the expansion of our current knowledge on our microbial companions, providing new valuable insights on microbiota variation and its impact on human health, on a large scale (Clarke et al., 2014).

1.5.1. The microbiome-gut-brain axis

The gut-brain axis comprehends the bidirectional communication network between the ENS and the CNS. In the last decades, the gut microbiome has been implicated as a critical element on this axis as many bacteria are capable of synthetizing or recognizing the same neurochemicals as those produced by the host nervous system and thus possibly influencing the host CNS through immunologic, biochemical, or neuroendocrine mechanisms. The noninfectious ability of these microbes to influence the host behavior has been reported in multiple studies. Specific gut bacteria have been shown to cause specific host behavioral changes. The first succeeding study in that field consisted of oral administration of *Campylobacter jejuni* in mice, which resulted in anxiety-like behavior, through vagus nerve communication (Lyte, 2013). Germ-free mice show development and physiologic irregularities, which are reversible by early life colonization with gut bacteria. Stress responses also seem to be affected by the gut microbiome as studies with germ-free mice showed that they were less timid and vigilant comparatively to conventional mice, affecting their survival instincts (Galland, 2014).

Overall, there may be three major ways by which the gut microbiome communicates with the human brain. The first involves signaling via the vagus nerve, which is thought to be stimulated by gut microbe-produced neurotransmitters, such as serotonin, and cause activity alterations in specific brain regions and consequently affect multiple body processes. The second is that the gut microbes stimulate immune cells to release cytokines into the bloodstream, ultimately influencing neurological processes in the CNS, such as microglia activation. The third hypothesis is the combination of the previous two – the gut microbe-produced metabolites (which include neurotransmitters, fatty acids, and other molecules) may travel to the brain via bloodstream or stimulate gut cells to produce compounds that will activate the vagus nerve (Kanwal, 2016).

In addition to the effects that gut microbes exert in the human organism, the gut microbiota itself is also affected by the host and interacts with host-derived compounds. This duality of influencing and being influenced by the host, leads the microbiota to take part in the regulation of complex endocrine networks, virtually functioning as an "endocrine organ" (Clarke et al., 2014). However, the molecular cascades involved in these events are still being investigated and, so far, a lot of important information has been acquired (Kanwal, 2016). For instance, the microbiome-gut-brain interaction has been implicated in multiple CNS and ENS related disorders such as ASD, anxiety, depression, Parkinson's disease, and irritable bowel syndrome (IBS) (Israelyan & Margolis, 2019).

1.5.2. **The microbiome-gut-brain axis and the serotonergic system**

Current studies have been revealing the direct and indirect effects of gut bacteria activity on tryptophan metabolism and the serotonergic system (O'Mahony et al., 2014) and although it has been determined that gut microbes play a very important role in their regulation, the exact mechanisms through which that occurs are still to be elucidated (Yano et al., 2015). Those effects have been strongly hypothesized to occur through bacterial manipulation of the host serotonergic system pathways and the levels of its intervening molecules, particularly tryptophan (Knecht, 2016). It has been previously evidenced that germ-free mice have low blood and colon levels of serotonin. Some microbial secondary metabolites impact gut serotonin levels, such as short-chain SFCA, which induce TrpH 1 transcription in EC cells (Yano et al., 2015), and fecal metabolites produced by *Clostridium* species, which seem to increase serotonin levels in EC cell cultures and in germ free mice (Israelyan & Margolis, 2019). The bacterial use of tryptophan in alternative dominant metabolic processes, such as the kynurenine pathway, may also be responsible for decreasing tryptophan availability in the host's gut (Yano et al., 2015). This is supported by studies with germ-free mice where elevated plasma tryptophan levels were observed, which could be normalized after gut microbial colonization (Clarke et al., 2014).

1.6. Serotonin biosynthesis in bacteria

Although the physiological roles of serotonin and melatonin in bacteria are not fully understood yet, they appear to be mostly related to defense mechanisms. Serotonin has been implicated in intercellular communication between microorganisms, growth regulation and protection against UV radiation. Exogenous serotonin also seems to be important in biofilm formation in *Pseudomonas aeruginosa* through quorum sensing mechanisms. Melatonin's main

role in microbes seems to be related to antioxidant protection, and it also seems to contribute to circadian rhythm regulation in certain human gut bacteria species, as dark conditions seem to cause increasing levels of this molecule. As these indolamines have demonstrated such beneficial effects in microbial survival, it would be evolutionarily favorable for these organisms to retain the capacity to synthetize such important compounds (Danilovich, Alberto, & Juárez Tomás, 2021).

While the serotonin and melatonin biosynthetic pathways for animals and plants are well established, there is still not a scientific consensus for these mechanisms in bacteria. *De novo* serotonin production has been theoretically reported in some bacteria species such as *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptococcus thermophilus* (Ozogul et al., 2012), *Morganella morganii*, *Klebsiella pneumoniae* and *Hafnia alvei* (Ozogul, 2004). Most of the studies that report bacterial serotonin production are based on detection of serotonin (most of the cases, in very low quantities) and/or its biosynthetic intermediaries in the culture medium, which is not considered 100% reliable evidence of it being, in fact, synthesized by the bacteria strains. Thus, despite all the efforts that have been made for decades now, there is still no solid evidence of bacterial serotonin biosynthesis, as the data available on this topic is not very extensive and some of the existent literature is ambiguous or vague. To date, we could only find one report that came close to demonstrate serotonin biosynthesis by bacteria. This study by Ma *et al.* (2017) used isotope-labeled L-tryptophan to characterize the melatonin biosynthetic pathway in *Pseudomonas fluorescens*, which resulted in detection of isotope-labeled serotonin by HPLC-MS. Isotope-labeled 5-hydroxytryptophan, but not tryptamine, was also detected, suggesting that this specific strain possibly produces serotonin through an identical pathway to the one observed in vertebrates and therefore does not use AAAD in these reactions. However, the bacterial genes involved in this pathway were not investigated in the study and the tryptophan hydroxylase gene was not found in the previously published genome sequences of *P. fluorescens*, possibly indicating that either the gene is contained in a plasmid or the tryptophan hydroxylation reaction is catalyzed by a different enzyme, such as phenylalanine hydroxylase (Ma et al., 2017), which has been previously confirmed to hydroxylate tryptophan in *P. fluorescens* (Lin et al., 2014). Tryptamine, the product of tryptophan decarboxylation in plants, has been detected in cultures of some bacteria species such as *Clostridium sporogenes*, *Ruminococcus gnavus* (Williams et al., 2014), *Hafnia alvei*, *Morganella morganii* and *Klebsiella pneumoniae* (Ozogul, 2004), possibly indicating a plant-alike serotonin biosynthetic pathway in these strains, even though it is not totally clear if this compound was indeed a product of the bacteria metabolism.

1.6.1. Aromatic amino acid decarboxylases and aromatic amino acid hydroxylases in bacteria

Biogenic amines (BA), which include serotonin, tyramine, tryptamine, and melatonin, are mainly produced through amino acid decarboxylation, and therefore, a great part of the currently existing bacterial AAAD research was performed in the scope of BA production in fermented foods, mostly in lactic acid bacteria, due to their adverse effects on human health. To date, it is known that at least some of the bacterial decarboxylase genes involved in BA synthesis are located in genomic islands or in unstable plasmids. Additionally, these genetic elements appear to be strain specific rather than species specific. Such observations led scientists to believe that the BA-producing capability of bacteria is a result of horizontal gene transfer that occurred between microbial organisms throughout evolution (Mohedano et al., 2015). This research field has been mainly focused on developing methods to effectively detect BA-producing strains in food. Tyramine, the product of tyrosine decarboxylation by tyrosine decarboxylase, is one of the most studied BA in this context due to its dangerous effects on the vascular system when ingested (Mohedano et al., 2015), and the tyrosine decarboxylase (tdc) gene has already been identified in multiple bacterial strains, such as *Enterococcus faecalis* (Pessione et al., 2009), *Enterococcus faecium, Enterococcus durans* (Burdychova & Komprda, 2007), *Lactobacillus lactis*, *Lactobacillus brevis* (Fernández, Linares, & Alvarez, 2004) and *Carnobacterium divergens* (Coton et al., 2004). On the other hand, tyramine production has been reported in other bacteria genera, such as *Micrococcus* (Nakazawa, Kumagai, & Yamada, 1977) and *Proteus* (De Llano, Cuesta, & Rodríguez, 1998), where no tyrosine decarboxylase genes have been identified to date.

Microbial AAA hydroxylases differ from the eucaryotic ones in the means that they are usually monomeric, while mammalian hydroxylases are polymeric, and lack an N-terminal extension of about 200 amino acids, as well as the C-terminal domain, which is involved in tetramerization in the eucaryotic enzymes (Zhao et al., 1994). Most of the AAAH bacterial genes discovered to date are annotated as phenylalanine hydroxylase genes. AAAH genes from *Pseudomonas aeruginosa* (Zhao et al., 1994), *Chromobacterium violaceum* (Chen & Frey, 1998), *Colwellia psychrerythraea* (Leiros et al., 2007) and multiple *Chlamydia* species (Abromaitis, Hefty, & Stephens, 2009) showed homology with mammalian hydroxylases. Some of these genes, specifically PheH genes, seem to be contained in operons along with other enzymes such as dehydratases and aminotransferases, in *P. aeruginosa* (Zhao et al., 1994), as well as transporters and regulatory proteins, in *P. putida* (Herrera & Ramos, 2007).

During the last decade, some studies reporting the possible mis annotation of some bacterial AAAD have been emerging. Koyanagi et al. (2012) reported high L-DOPA specificity for a *Pseudomonas putida* AAAD, whose gene was previously annotated as tyrosine decarboxylase gene (Koyanagi et al., 2012). In 2014, Williams and their team carried out enzymatic studies in AAAD isolated from *Clostridium sporogenes* and, even though the enzyme's genetic sequence was previously annotated as a tyrosine decarboxylase gene, their experiments showed that the enzyme was more efficient at decarboxylating tryptophan. However, the assay used in the study is only qualitative and some experimental issues regarding limited solubility of tyrosine are mentioned, which might have compromised the accuracy of the results (Williams et al., 2014). A tyrosine decarboxylase gene from *Lactobacillus brevis* was cloned and expressed, and demonstrated enzymatic activity with tyrosine and L-DOPA, but not glutamate, despite sharing 100% amino acid sequence identity with an AAAD previously annotated as glutamate decarboxylase from a different *L. brevis* strain (Zhang & Ni, 2014).

1.6.2. Bacterial AAAD and AAAH substrate promiscuity

As previously stated, throughout the last decades, a few studies have been reporting tryptamine and 5-hydroxytryptophan formation in bacteria. However, and in line with what is known about the serotonin biosynthetic pathways in eukaryotes, not many genes responsible for the conversion of tryptophan into these compounds, and ultimately into serotonin, have been identified in bacteria so far. Tryptophan decarboxylase activity is very uncommon among bacteria (Williams et al., 2014) and, to our knowledge, no bacterial tryptophan hydroxylase genes have been described. Furthermore, the simultaneous presence of AAAH and AAAD genes in the same bacterial strain is very rare. *Pseudomonas putida* KT2440 is one of those rare cases, in which both AAAH (Herrera & Ramos, 2007) and AAAD (Koyanagi et al., 2012) genes have been reported. However, the fact that serotonin biosynthetic intermediaries have been detected in some cultured strains while no tryptophan hydroxylase and/or decarboxylase genes are known to be present in their genome, may hint that these reactions might be carried out by other enzymes capable of using similar substrates. Additionally, the previously mentioned enzyme function mis annotation reports might be related to enzymatic substrate promiscuity in some cases, rather than actual specificity identification errors, as these enzymes may be able to catalyze different reactions.

Enzyme substrate promiscuity is not a new concept. The widely spread capacity of enzymes to use diverse substrates and catalyze different reactions other than those they are

specialized at, has been known for a long time. It has been theorized that primordial enzymes might have had very broad specificities, acting on multiple substrates, granting them an expanded metabolic range. Evolutionarily, enzymes became more and more specialized, which improved their metabolic efficiency, but the promiscuous substrate function found in present enzymes belonging to the same family might suggest their divergent evolution from a common ancestor (Khersonsky & Tawfik, 2010).

Eucaryotic AAAH and AAAD substrate promiscuity has been reported before. Because the biopterin-dependent AAAH share homologous catalytic cores and their aromatic substrates have relatively similar structures, it has been theorized that the three hydroxylases will use all three substrates, at least to some extent (Fitzpatrick, 1999). As for AAAD, although they all seem to be evolutionarily related, plant AAAD usually exhibit increased specificity, in contrast to animal AAAD, that typically seem to be able to use different substrates (Kawalleck et al., 1993). In bacteria, even though the research is not as extensive, current knowledge suggests that the substrate promiscuity is shared among bacterial enzymes as well. Overall, the evidence seems to hint that serotonin production in bacteria might occur through different enzymes other than those already described for animal and plants' classical biosynthetic pathways, particularly different AAAH and AAAD, which might then be able to use multiple substrates, and possibly taking part in different biosynthetic pathways.

Therefore, genomic studies are extremely important in this research field, to find bacterial genes that encode enzymes possibly involved in serotonin and melatonin synthesis, particularly aromatic amino acid hydroxylases (AAAH) and aromatic amino acid decarboxylases (AAAD), which are the two key enzymes in the eukaryotic serotonin biosynthetic pathway (Figure 4).

1.7. Objective

The main focus of this project is exploring the serotonin biosynthetic pathways in bacteria, using *Pseudomonas putida* KT4220 as a model. As mentioned before, serotonin biosynthesis has been previously detected in members of the *Pseudomonas* genera (Ma et al., 2017), and both AAAH (Herrera & Ramos, 2007) and AAAD (Koyanagi et al., 2012) genes have been identified in *P. putida*. Since most bacterial AAAH are identified as phenylalanine hydroxylases, the aim of this study was to test the substrate specificity of a *P. putida* AAAH previously annotated as PheH, i.e., if this enzyme could also hydroxylate tyrosine and tryptophan or if it was specific for phenylalanine.

Seeing the impact our gut microbiota composition has on so many physiological processes, certain pathologic processes may be influenced by the presence of specific microbes or microbe-derived metabolites in the human gut as well, particularly serotonin-producing bacteria and serotonin (and its precursors).

Even though *P. putida* is a soil bacterium (Belda et al., 2016), testing its AAAH and AAAD specificity, particularly their hypothetical tryptophan hydroxylation and decarboxylation capacity, may open the path to finding homologues in our gut bacterial communities, where this metabolic function may be very important.

In line with these hypotheses, a synthetic AAAH gene from *P. putida* was cloned. The recombinant enzyme was produced and purified, and enzymatic activity tests using different substrates and biochemical conditions were then performed to probe its suspected promiscuity towards different AAA.

Chapter 2 – Materials and Methods

2.1. Synthetic gene acquisition, heterologous expression, and enzyme purification

The amino acid sequence (AAN70065.1) encoded by the putative AAAH gene from *Pseudomonas putida* KT2440 was retrieved from the National Center for Biotechnology Information database, through BLAST searches (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Based on the previously selected amino acid sequence, a synthetic gene, codon-optimized for expression in *Escherichia coli*, was acquired (Genscript Corp.). The gene was cloned into the Nde I and Sap I restriction sites of the expression vector pTXB1 (New England Biolabs), which results in the fusion of a mini-intein (*Mxe* GyrA intein) tag, containing a chitin-binding domain, to the C-terminus of the protein, necessary for the protein purification steps. The recombinant vector was transformed into *E. coli* BL21(DE3) chemically competent cells. The expression and purification protocols were performed according to IMPACT kit instructions (New England Biolabs #E6901S). Briefly, a clone carrying the recombinant plasmid was grown in 2 L of LB medium to an OD_{610}=0.6 and expression was induced with 0.5 mM IPTG. Cultures were kept overnight at 20°C in an orbital shaker at 120 rpm. After overnight culture, the cells were centrifuged at 9000 x g for 10 minutes at 4°C and the supernatant discarded. The cell pellet was resuspended in 30 mL of ice-cold Column Buffer and the cells were lysed by sonication on ice. The lysate was then centrifuged at 14000 x g for 30 minutes at 4°C to remove cell debris and obtain the soluble cell extract.

For the affinity purification procedure, the chitin column (which binds the chitin-binding domain (CBD) in the fusion protein intein-tag) was equilibrated with Column Buffer and the cell extract was loaded at a flow rate of 0.5-1 mL/min. The column was washed to eliminate nonspecific binding of other proteins. On-column cleavage was induced by adding Cleavage Buffer (Annex 1) to the column, followed by incubation at 46°C for 15 hours. The protein was then eluted from the column using Column Buffer (Annex 1), causing the intein-CBD tag to remain bound to the resin. The cleavage efficiency and purity of the eluted fractions were examined by SDS-PAGE analysis. Finally, the purest fractions were pooled, concentrated by ultrafiltration, and equilibrated with 50 mM BTP buffer pH 7.5 and stored at -80°C.

The gene cloning and expression experiments described in this section (section 2.1) were carried out by Daniela Costa back in 2018 and, therefore, the results will not be included in this report.

2.2. Sequence analysis, phylogenetic tree and protein three-dimensional structure model

The amino acid sequence of the putative AAAH (PP_4490) was used in a BLAST search for homologues in other species, in the Universal Protein Resource database (UniProt,

www.uniprot.org/blast). The selected amino acid sequences were aligned using Clustal Omega (Sievers et al., 2011) and analyzed using ALINE (Bond & Schüttelkopf, 2009).

For the phylogenetic analysis, the selected amino acid sequences were first aligned (ClustalW alignment system) and a phylogenetic tree was then generated (applying the Maximum Likelihood statistical method and the Jones-Taylor-Thornton matrix-based model), using MEGA version X (Kumar et al., 2018).

A three-dimensional structural model of the PP_4490 was generated based on its closest structural homologue model, using SWISS-MODEL (Waterhouse et al., 2018).

2.3. Analysis of enzyme purity by SDS-PAGE

The recombinant protein, previously stored at -80°C, was analyzed through SDS-PAGE to confirm it was still pure and did not undergo degradation. The Resolving Gel and Stacking Gel solutions (Annex I) were prepared and left to polymerize. Loading buffer (5x SDS-PAGE Sample Loading Buffer, NZYTech) and 50 mM BTP pH 7.5 were added to the protein samples (4 µg and 6 µg of enzyme) to bring them to a total volume of 10 µL and incubated at 98°C for 5 minutes. The samples were then loaded into the gel wells, along with a low molecular weight protein marker (NZYTech). The Running Buffer (Annex I) was added to the system and the electrophoresis was set to run at 200 V for 45 minutes. Finally, the gel was stained with BlueSafe (NZYTech) for 20 minutes, to observe the bands.

2.4. Analysis of enzyme substrate specificity by thin layer chromatography

Thin-layer chromatography (TLC) analysis was performed to test enzyme activity with L-tryptophan, L-phenylalanine, and tryptamine. The analysis was performed in a 50 µL mixture containing 0.5 µL of pure enzyme 4.5 µg/µL, 3.3 µL of 30 mM substrate (L-Trp, L-Phe or Try), 5 µL of 50mM BH_4, 5 µL of 100 mM DTT pH 7.5 and 1.6 µL of 15mM $FeSO_4$. Equivalent mixtures without the enzyme were used as negative controls. 20 µL standards of 2 mM L-tryptophan, 5-hydroxytryptophan, L-phenylalanine, L-tyrosine, tryptamine, and serotonin were also included. All reactions were carried out in 100 mM BTP pH 7.5 buffer at 30°C for 30 minutes.

20 µL of each reaction, as well as the corresponding controls and standards, were subsequently spotted into a silica gel plate (Silica Gel 60, Merck) and incubated in a covered glass chamber with ethyl acetate/isopropanol/ammonium 25% (45:35:20, v/v/v) solvent system for approximately 2h. After incubation, the chromatogram was dried with a hot stream of air for

40 minutes. The plate was then sprayed with a ninhydrin solution (Annex 1) and heated at 120°C for 3 minutes, to make the substrates and products' spots visible.

2.5. Enzyme activity assays

2.5.1. Enzyme activity with phenylalanine

Quantification of tyrosine production was done according to a previously described method, through fluorescence spectroscopy analysis (Gersting et al., 2010).

Before enzyme activity experiments, standards consisting of different concentrations of L-tyrosine (0-175 µM) in a mixture containing 550 µM L-phenylalanine, 40 mM NaHEPES pH 7.3, 1 mg/mL catalase (Sigma-Aldrich), 10 µM ferrous sulfate ($FeSO_4$) and 75 µM BH_4, were loaded into an opaque 96-well microplate (Thermo Scientific Nunc F96) and fluorometrically measured (Molecular Devices SpectraMax Gemini EM Microplate Reader) at an excitation wavelength of 274nm and emission wavelength of 304nm. A standard curve was constructed using these fluorometric measurements. All individual experiments were performed in duplicate and in a final volume of 200 µL.

For the quantification of tyrosine production, a mixture containing 40 mM NaHEPES pH 7.3, 1 mg/mL catalase, 10 µM $FeSO_4$, 1 mM L-phenylalanine and 0.01 mg/mL enzyme, was prepared. A blank reaction consisting of the same conditions and without the enzyme was used as negative control. After loading the mixtures into the microwell plate (Thermo Scientific Nunc F96), the reactions were initiated by adding 75 µM BH_4 to the plate wells. Time-dependent L-tyrosine production was assessed by fluorometric measurements (Molecular Devices SpectraMax Gemini EM Microplate Reader) at an excitation wavelength of 274nm and emission wavelength of 304nm, at 25°C for 5 minutes (with readings every 8 seconds). All individual experiments were performed in duplicate and in a final volume of 200 µL.

Blank reactions were subtracted to the enzymatic reactions and the corresponding enzyme activity values (nmol Tyr/min x mg protein units) were determined for each experiment, using the standard curve obtained previously.

2.5.2. Enzyme activity with tyrosine

Quantification of L-DOPA production was done according to a previously described method, through spectrophotometry analysis (Vermeer et al., 2013).

Two mixtures (A and B) were prepared separately. Mixture A was prepared using 10 mM HEPES buffer, 10 µg/µL enzyme, 0.25 mM BH_4 and 2.5 µM $FeSO_4$, and incubated in ice for 5-10 minutes, to allow the binding of the iron and the BH_4 to the enzyme. Mixture B was prepared with 10 mM HEPES buffer, 50 µM tyrosine and 100 µM sodium periodate. A negative control (without the enzyme) and a positive control (with 50 µM L-DOPA, instead of tyrosine) with similar conditions were also included in the experiment.

For each condition, after Mixture A incubation, the two mixtures were combined in a 1:1 ratio and transferred to a 96-well microplate. The plate was immediately placed in the plate reader (Molecular Devices SpectraMax Plus 384 Microplate Reader) and spectrophotometrically measured at an absorbance of 475 nm, at 37°C for 5 minutes (readings every 10 seconds). All individual experiments were performed in duplicate and in a final volume of 200 µL.

2.6. Partial biochemical characterization of phenylalanine hydroxylase activity

Quantification of tyrosine production under different conditions of temperature, pH and $FeSO_4$ concentration, was assessed through fluorescence spectroscopy analysis, using the tyrosine quantification method described in section 2.7.1 (Gersting et al., 2010).

The temperature profile was determined between 25 and 40°C in assay mixtures with 40 mM NaHEPES pH 7.3, 10 µM $FeSO_4$ and 1 mM Phe. The pH profile was determined at 25°C in 40 mM NaHEPES (pH 6.9 – 8.0), 10 µM $FeSO_4$ and 1 mM Phe. The effect of $FeSO_4$ concentration on enzyme activity was determined between 0 µM and 100 µM of $FeSO_4$, at 25°C in 40 mM NaHEPES pH 7.3 and 1 mM Phe.

Chapter 3 – Results

3.1 Sequence analysis, phylogenetic tree and protein three-dimensional structure model

After BLAST searches for homologues, amino acid sequences of multiple AAAH were selected and aligned, along with the PP_4490 amino acid sequence (Figure 5), to analyze their differences and the similarities, particularly in the catalytic domain.

The catalytic domain (indicated by the purple line above the sequences, in Figure 5) is where most conserved residues are observed. The iron binding residues (two histidine and one glutamic acid, signaled by yellow stars in Figure 5) are strictly conserved in all AAAH. The residues that interact with the pterin cofactor (signaled by pink arrows in Figure 5), or at least their properties, are generally conserved as well, as the pterin-enzyme interactions are responsible for conformational changes that are essential to substrate binding and subsequent hydroxylation. Multiple other residues seem to be critical to these enzyme conformational changes as well, such as Tyr113 and Ser183 (signaled by blue arrows). Moreover, the residues that form the hydrophobic cage that surrounds the substrate aromatic side chain (signaled by green arrows in Figure 5) are also highly conserved between different AAAH. One exception is the Trp161 residue (in PP_4490) which is substituted for Phe in tryptophan hydroxylases, as shown in the *Gallus gallus* TrpH 1 sequence. This residue has been shown to be involved in discriminating between different aromatic substrates by site-direct mutagenesis studies. Studies with human TrpH 1 demonstrated that substitution of this Phe residue for a Trp residue (which is present in all PheH and TyrH, including the PP_4490 sequence) at that position, yielded a TrpH 1 variant with an even higher affinity for phenylalanine than human PheH itself (Skjærven, Teigen, & Martinez, 2014). However, substrate specificity seems to be the mostly affected by differential second sphere residue packing against the substrate catalytic cage (Fitzpatrick, 2003).

Overall, the resulting alignment (Figure 5) clearly shows that the eukaryotic AAAH sequences (*Gallus gallus* TrpH 1, *Caenorhabditis elegans* PheH and *Rattus norvegicus* TyrH) are longer than the prokaryotic ones, as the latter have shorter putative regulatory domains (in the N-terminus). This characteristic is thought to impact the catalytic efficiency as studies with human PheH showed that these longer regulatory domains often extend over the active site, potentially limiting ligand access (Erlandsen et al., 2002). Additionally, these regulatory domains have very low homology between different AAAH, which is consistent with the highly variable regulatory mechanisms reported for different enzymes. The final C-terminal residues are also highly dissimilar between species, as these residues are usually involved in protein tetramerization, which is characteristic of eukaryotic AAAH, as bacterial AAAH are mostly monomers (or dimers) (Fitzpatrick, 2003).

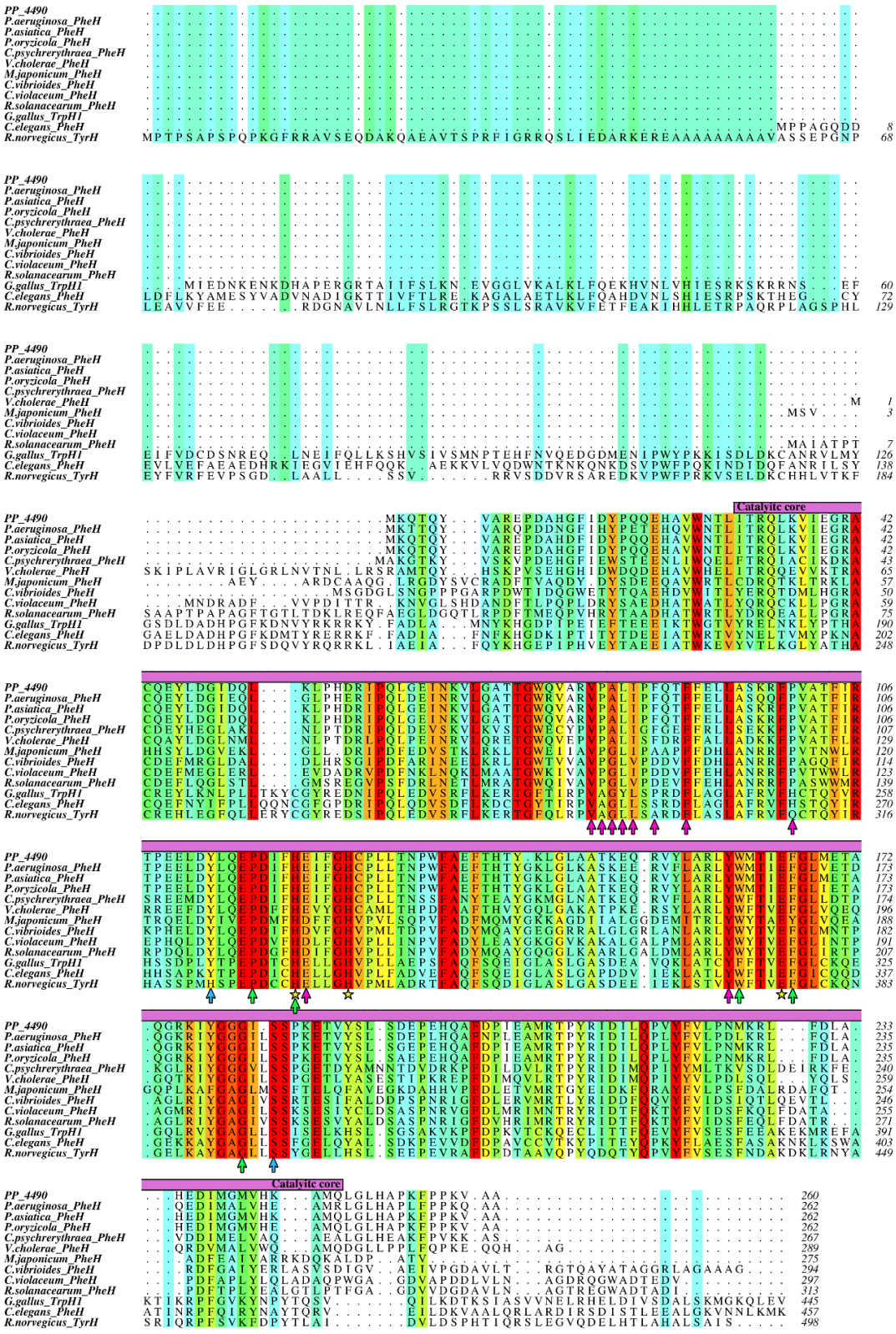

Figure 5. Amino acid sequence alignment of aromatic amino acid hydroxylases. Multiple bacterial phenylalanine hydroxylases' sequences were included (*Pseudomonas aeruginosa, Pseudomonas asiatica, Pseudomonas oryzicola, Colwellia psychrerythraea, Vibrio cholerae, Mesorhizobium japonicum, Caulobacter vibrioides, Chromobacterium violaceum* and *Ralstonia solanacearum*), as well as an outgroup comprising three eukaryotic AAAH (*Caenorhaditis elegans* PheH, *Gallus gallus* TrpH and *Rattus norvegicus* TyrH). Residues are colored based on a residue conservation scale (red: identical residues; orange to blue: decreasing conservation of amino acid properties; white: dissimilar residues). Yellow stars signal iron-binding residues; pink arrows signal residues

involved in enzyme-pterin cofactor interaction; green arrows signal residues that form the hydrophobic cage that surrounds the substrate aromatic side chain; blue arrows signal other important catalytic residues. The alignment was done using Clustal Omega (Madeira et al., 2019) and the figure was obtained with Aline software (Bond & Schüttelkopf, 2009).

For the phylogenetic analysis, the AAAH amino acid sequences were selected taking into consideration their similarity with the amino acid sequence of PP_4490. The generated phylogenetic tree is shown in Figure 6. The AAAH encoded by PP_4490 appears to be closely related to other *Pseudomonas* enzymes. No procaryotic TrpH or TyrH amino acid sequences were obtained in the BLAST searches and therefore were not included in the analysis. An outgroup composed by eukaryotic AAAH – PheH by *C. elegans*, TrpH 1 by *G. gallus* and TyrH by *R. norvegicus,* was included.

The three-dimensional model of the PP_4490 enzyme was generated using the already determined *Colwellia psychrerythraea* PheH structure as a template (2v27.1), with an amino acid sequence identity of 56,98% and a Global Model Quality Estimate (GMQL) of 84%. The protein structure model is shown is Figure 7(a) as a homodimer composed by two identical monomers. The orange spheres represent the iron ligands, which are located at the bottom of the active site cleft. The iron atoms are coordinated by three amino acid residues (two histidine and one glutamic acid), shown in Figure7(b).

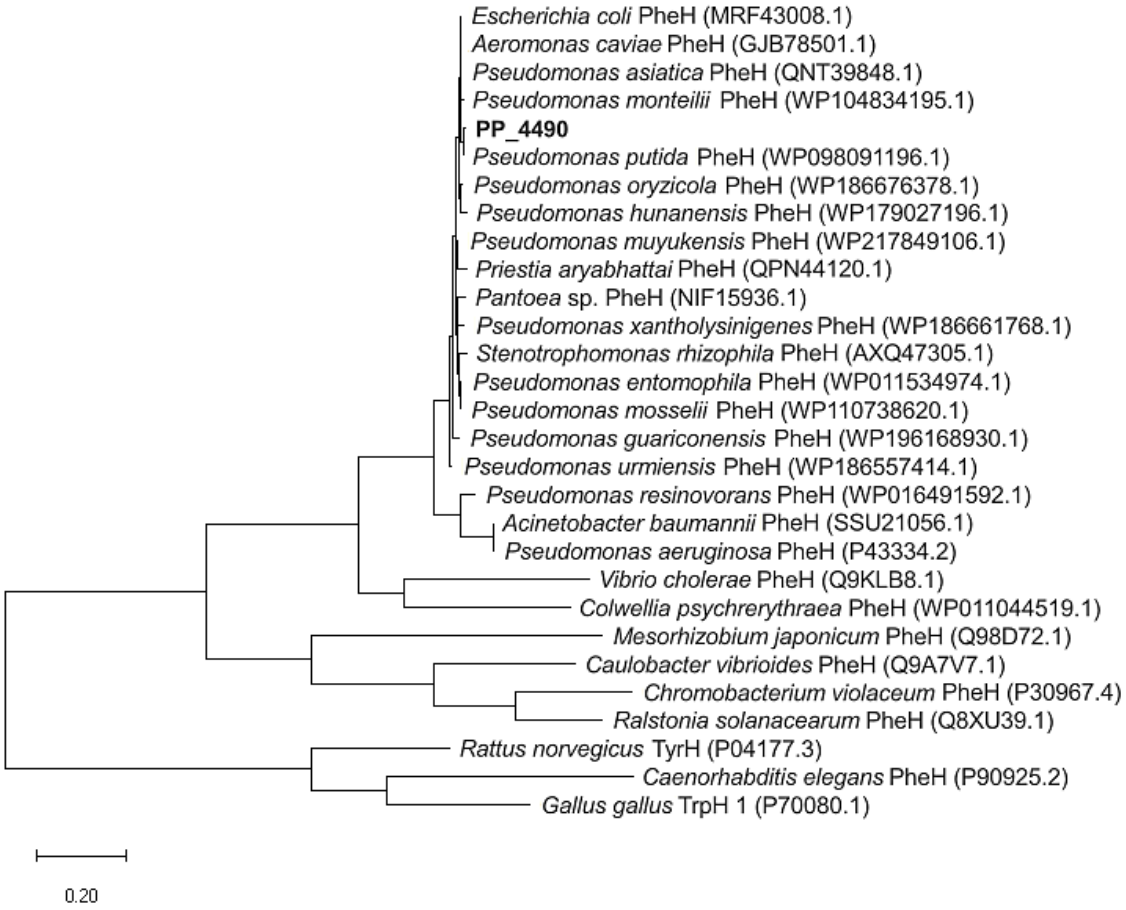

Figure 6. Phylogenetic tree of selected aromatic amino acid hydroxylases, based on amino acid sequence. The enzyme encoded by PP_4490 is in bold and is clustered with other *Pseudomonas* AAAH. The eukaryote AAAH outgroup (*Rattus norvegicus* TyrH, *Caenorhabditis elegans* PheH and *Gallus gallus* TrpH 1) is clearly separated from the prokaryotic enzymes. The phylogenetic tree generation (Neighbor joining clustering method) was carried out on MEGA version X (Kumar et al., 2018).

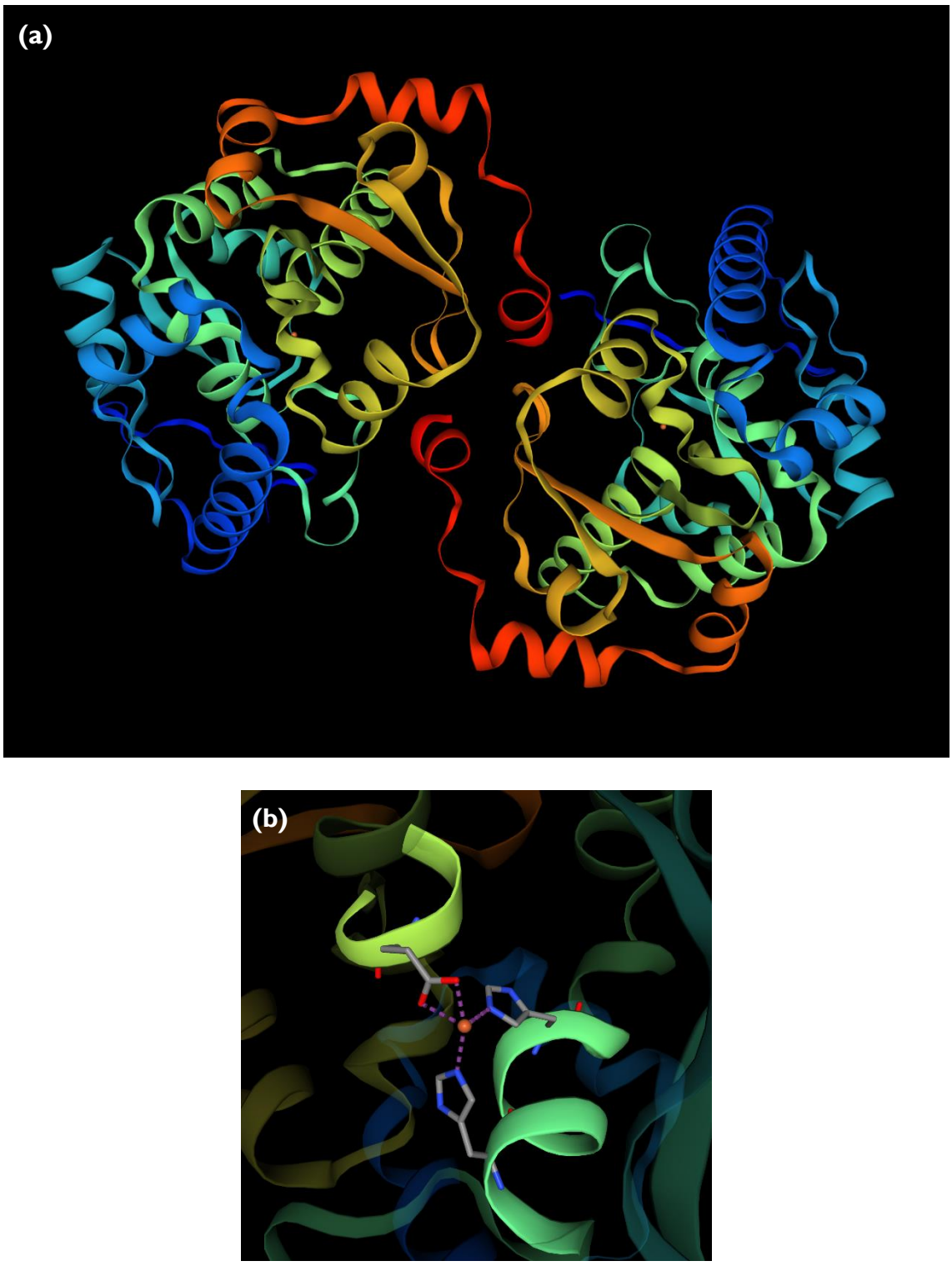

Figure 7. Three-dimensional protein structure homology model of the AAAH from *P. putida* KT2440. The model was build based on *Colwellia psychrerythraea* PheH template (2v27.1), with an amino acid sequence identity of 56,98% and a global model quality estimate (QMEANDisCo Global) of 0,78. (a) The protein is shown as a homodimer with two iron ligands (orange dots) that mark the location of the active site. (b) The iron atom is bound to three protein residues (two histidine and one glutamic acid). Figures were obtained using SWISS-MODEL (Waterhouse et al., 2018).

3.2. Analysis of enzyme purity by SDS-PAGE

The SDS-PAGE band profile is shown in Figure 8. No significant contamination or protein degradation were detected. The predicted molecular weight of the PP_4490 enzyme is ~30 kDa, which is consistent with the observed SDS-PAGE bands.

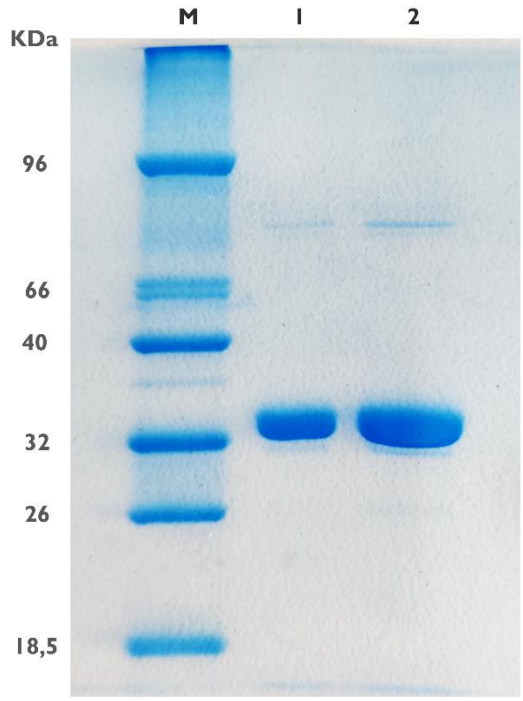

Figure 8. SDS-PAGE analysis of the pure recombinant PP_4490 enzyme. Lane M – molecular weight protein marker; Lane 1 and 2 – 4 µg and 6 µg, respectively, of purified recombinant protein.

3.3. Analysis of enzyme substrate specificity by Thin Layer Chromatography

The AAAH activity was tested with three different substrates (Tryptophan, Tryptamine, and Phenylalanine) through Thin Layer Chromatography (TLC) analysis. The results are shown in Figure 9. No AAAH activity was detected with Tryptophan or with Tryptamine as the 5-HTP and the serotonin spots (visible in the 5-HTP and serotonin standard lanes, respectively) are not detected in the Tryptophan (lane 2) and Tryptamine (lane 6) reaction lanes, respectively. The Tyrosine spot in the Phenylalanine reaction lane (lane 10) confirms the AAAH activity with this substrate.

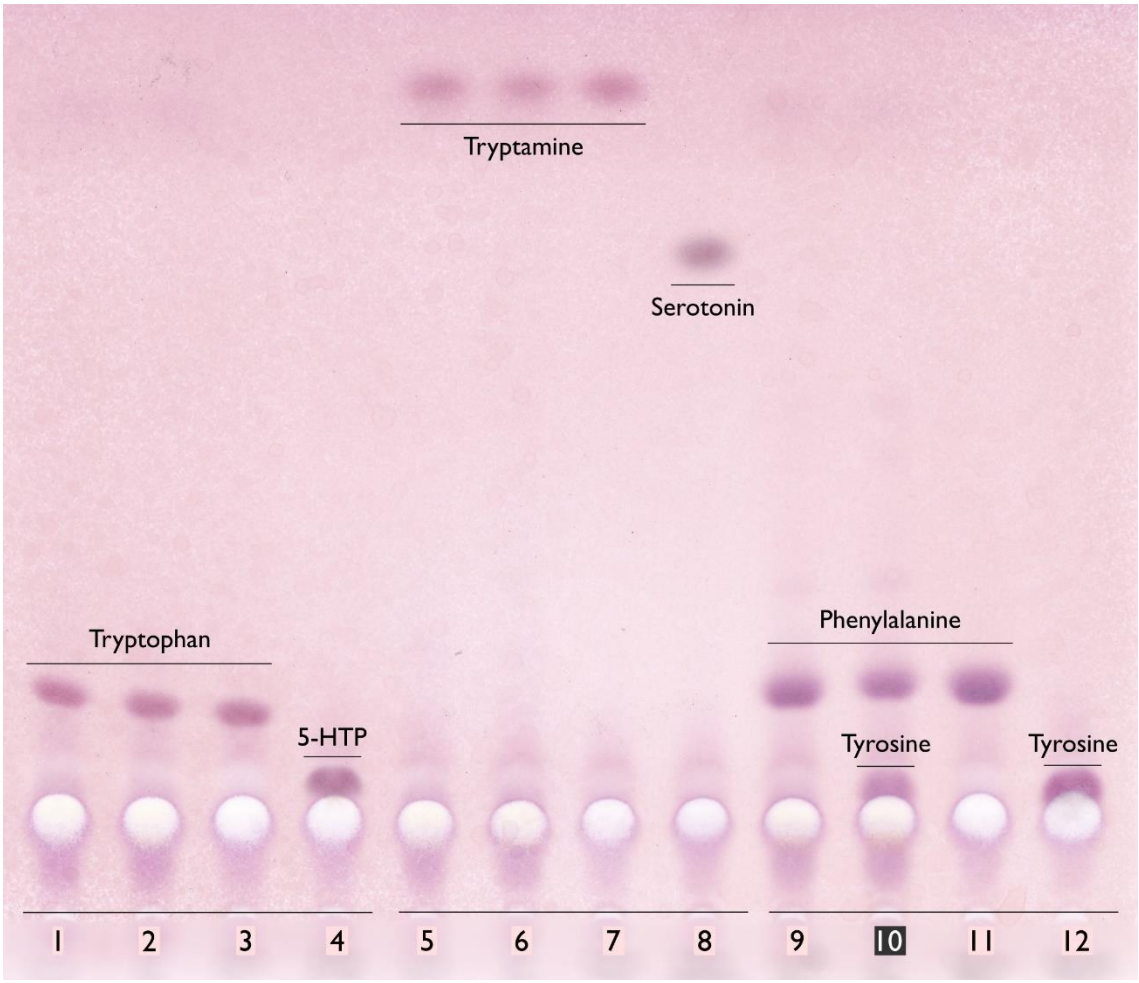

Figure 9. Thin-layer chromatography analysis of AAAH substrate specificity using ethyl acetate/isopropanol/ammonium 25% (45:35:20, v/v/v) solvent system. Lane 1, 5 and 9 – controls without enzyme; Lanes 2, 6 and 10 – reactions with enzyme using tryptophan (2), tryptamine (6) and phenylalanine (10) substrates; Lanes 3, 4, 7, 8, 11 and 12 – standards of tryptophan (3), 5-hydroxytryptophan (4), tryptamine (7), serotonin (8), phenylalanine (11) and tyrosine (12). Lane 10 (phenylalanine hydroxylation) is the only lane where an enzymatic reaction can be observed. In this solvent system, spots corresponding to aromatic amino acids and their hydroxylated products appear in a pink/purple color.

3.4. Enzyme activity assays

3.4.1. Enzyme activity with phenylalanine

The time dependent AAAH tyrosine production, detected through fluorescence spectroscopy analysis, is shown in the Figure 10 bar chart. Tyrosine fluorescence intensity increased through time (green bars), which suggests that the hydroxylase was effectively converting phenylalanine into tyrosine.

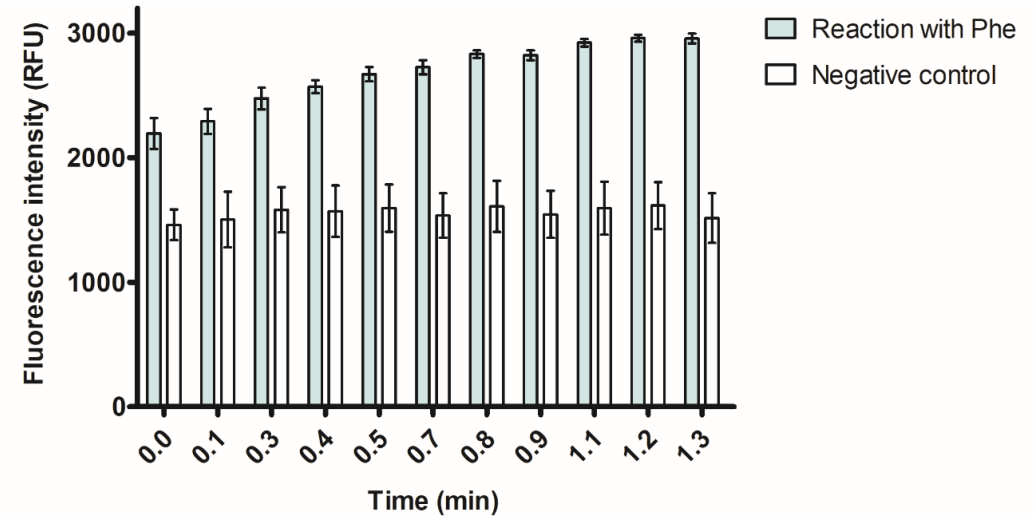

Figure 10. Time-dependent fluorescence intensity (RFU) measurement of AAAH reaction with phenylalanine substrate. The negative control consisted of the same conditions, without the enzyme. Fluorometric measurements were performed at an excitation wavelength of 274nm and emission wavelength of 304nm, at 25°C for 5 minutes (with readings every 8 seconds), in a Molecular Devices SpectraMax Gemini EM Microplate Reader.

3.4.2. Enzyme activity with tyrosine

The time dependent AAAH activity with tyrosine was accessed by absorbance measurements at a wavelength of 475 nm, for 5 minutes. As shown in the Figure 11 bar chart, no hydroxylase activity was detected 5 minutes after reaction initiation, which implies that the AAAH was not capable of hydroxylating tyrosine (in these experimental conditions).

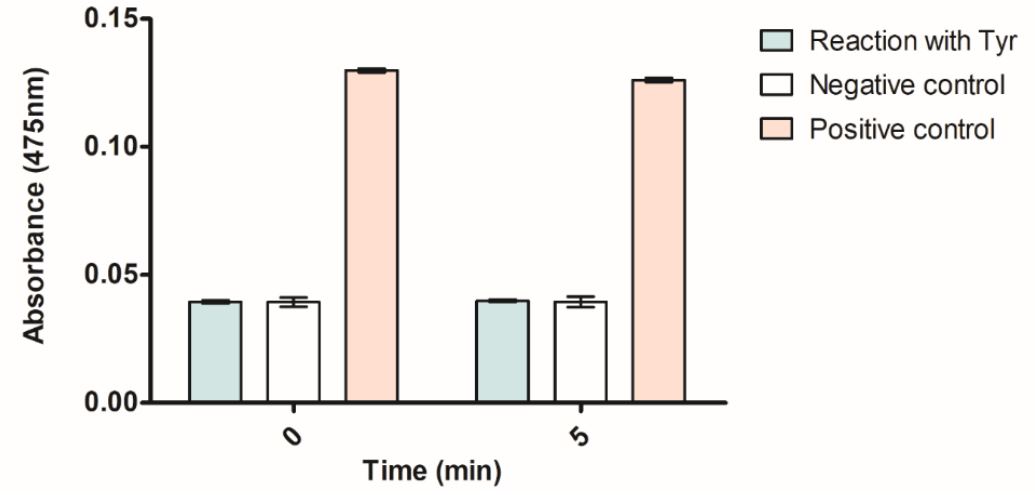

Figure 31. Time-dependent absorbance measurement of AAAH reaction with tyrosine substrate. The negative control consisted of the same conditions as the reaction, but without the enzyme. The positive control consisted of the same conditions as the reaction, but without the enzyme and with L-DOPA instead of tyrosine. The absorbance values did not vary significantly throughout the measurement time. Spectrophotometric measurements were performed at a wavelength of 475nm, at 37°C for 5 minutes (with readings every 10 seconds), in a Molecular Devices SpectraMax Plus 384 Microplate Reader.

3.5. Partial biochemical characterization of phenylalanine hydroxylase activity

Given the previously obtained results, AAAH activity with phenylalanine was tested in different temperature and pH conditions, as well as with different concentrations of iron.

For the temperature profile, shown in Figure 12, AAAH activity was fluorometrically measured at 25°C, 30°C, 35°C and 40°C. The activity decreased as temperature increased, which indicates that the maximum enzymatic activity occurs at 25°C (or lower, as temperatures under 25°C were not tested due to equipment function limitation).

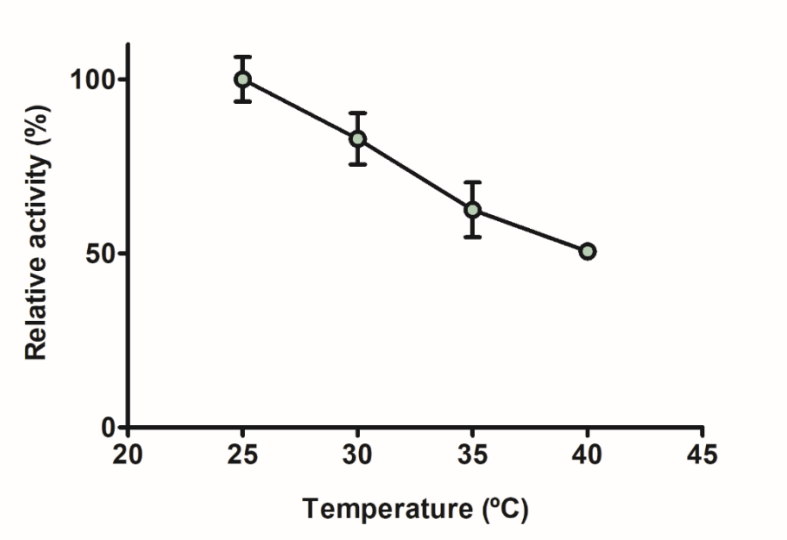

Figure 12. Effect of temperature on AAAH activity at pH 7.3 in the presence of 10 µM $FeSO_4$. Activity values (nmol Tyr/min x mg protein units) were calculated for each experiment, using the standard curve obtained initially and converted into percentages (relative activity). Fluorometric measurements were carried out at an excitation wavelength of 274nm and emission wavelength of 304nm using a Molecular Devices SpectraMax Gemini EM Microplate Reader.

The pH profile was accessed fluorometrically at 25°C in the presence of 10 µM $FeSO_4$, at pH 6.9, pH 7.3, pH 7.6 and pH 8.0 (Figure 13). The AAAH was maximally active at pH 7.3, closely followed by pH 7.6. At lower (pH 6.9) and higher (pH 8.0) pH the activity decreased considerably.

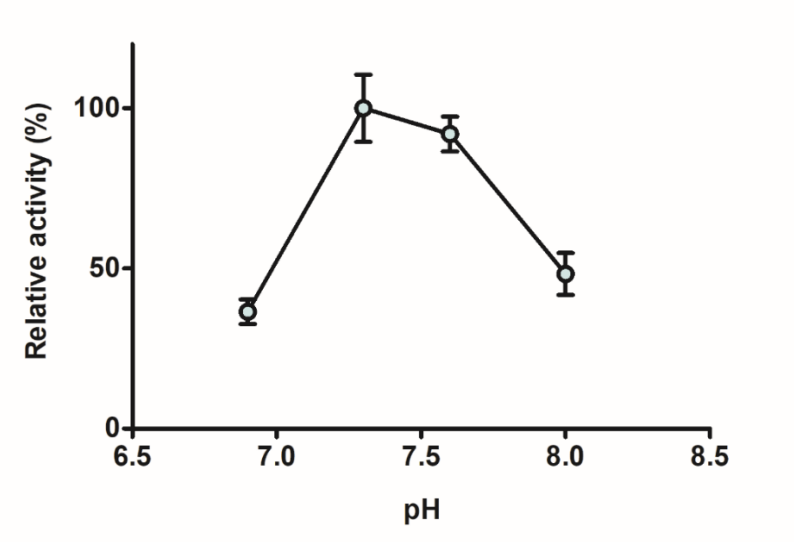

Figure 13. Effect of pH on AAAH activity at 25ºC in the presence of 10 µM $FeSO_4$. Activity values (nmol Tyr/min x mg protein units) were calculated for each experiment, using the standard curve obtained initially and converted into percentages (relative activity). Fluorometric measurements were carried out at an excitation wavelength of 274nm and emission wavelength of 304nm using a Molecular Devices SpectraMax Gemini EM Microplate Reader.

AAAH activity was tested using different concentrations of $FeSO_4$, between 0 µM and 100 µM (Figure 14). As enzyme activity was still being detected in the absence of $FeSO_4$, AAAH activity was also tested in the presence of EDTA, to bind the residual iron naturally present in the mixture. The maximum AAAH activity was detected at 5 µM $FeSO_4$, which was very close to the one detected with 10µM $FeSO_4$. Enzyme activity with iron concentrations under 0,1 µM was barely detectable. Iron concentrations above 10 µM resulted in lower enzyme activity, but still relatively high (above 75%). Concentrations above 100 µM were not tested as they caused iron precipitation.

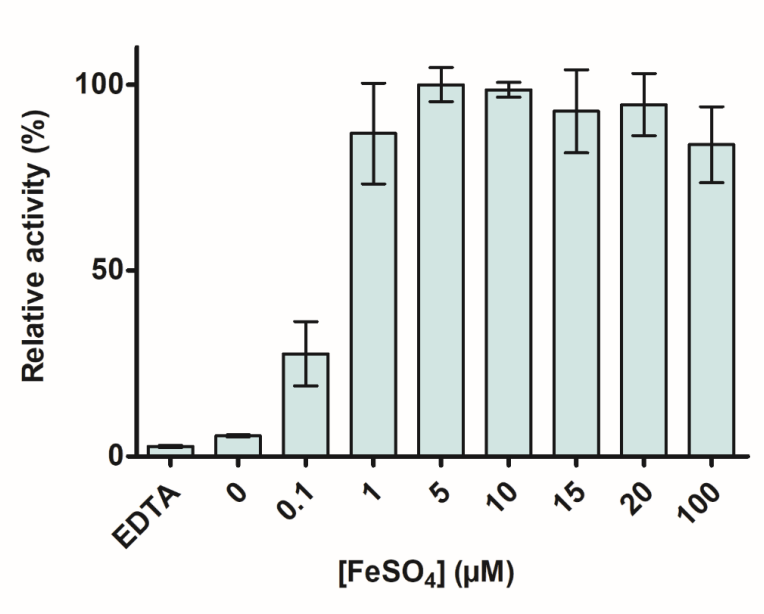

Figure 14. Effect of $FeSO_4$ concentration on AAAH activity at 25°C and pH 7.3. Activity values (nmol Tyr/min x mg protein units) were calculated for each experiment, using the standard curve obtained initially and converted into percentages (relative activity). Fluorometric measurements were carried out at an excitation wavelength of 274nm and emission wavelength of 304nm using a Molecular Devices SpectraMax Gemini EM Microplate Reader.

Chapter 4 - Discussion

Serotonin and melatonin are two key neuroactive compounds in many vital metabolic processes. Disruption of their levels, and the molecular pathways in which they intervene, has been implicated in the pathophysiology of several human disorders, as they impact multiple mechanisms, in both neurological and peripheral processes. Serotonergic dysfunction, particularly, has been linked to disorders like depression, anxiety, OCD, ADHD (Pourhamzeh et al., 2021), fibromyalgia (Welsch et al., 2018), PTSD (Zhao et al., 2017), suicidal behavior, bipolar disorder (Giurgiuca et al., 2017), IBS, obesity, neuroendocrine tumors (Banskota, Ghia, & Khan, 2018). For this reason, throughout the last couple of decades, continuous efforts have been made to develop innovative therapies based on these compounds and their intermediates. Multiple pathologies are already being treated using these indolamines as both therapeutic agents and pharmacological targets (Danilovich, Alberto, & Juárez Tomás, 2021).

Currently, melatonin is industrially produced through chemical synthesis, which is a complex process that often requires toxic substrates and catalysts. Microbial production of these beneficial indolamines has been picking up interest among the scientific community as it is a safer and an ecologically more sustainable approach. Even though these "microbial cell factories" usually rely on genetically modified organisms with synthetically optimized metabolisms (Sun, Chen, & Zhang, 2016), using wild-type microorganisms capable of naturally producing these compounds is an interesting subject to further explore. The possible existence of microbial species capable of producing serotonin and melatonin in our gut microbiome is an innovative research topic. Taking all the recent data on the microbiome-gut-brain communication into account, these putative indolamine-producing gut microbes may prove to be therapeutically very relevant.

However, there is still a long way to go as the microbial serotonin and melatonin biosynthetic pathways are still to be fully elucidated (Danilovich, Alberto, & Juárez Tomás, 2021). Taking all the information previously discussed in Section 1.6 (Introduction) together, so far, evidence points to similar pathways as the ones described for eukaryotes, as eukaryote-like AAAH and AAAD (as well as their genes) and some of the intermediary compounds, have been discovered in multiple bacteria, even though serotonin production has not yet been effectively detected in most species.

Tryptophan hydroxylase, particularly, has not been reported in bacteria yet (Williams et al., 2014). However, other AAAH have been effectively identified in some of these organisms and, even though different AAAH may have different substrate specificities, the fact that their substrates are structurally very similar and that they require the same cofactors (iron and pterin), leads to the possibility that these enzymes can hydroxylate all three aromatic amino acids, at least to some extent, which may impart some level of substrate promiscuity,

which we sought to explore in this work. Additionally, even though the regulatory domain has been implicated in substrate specificity as well (particularly in catalytic efficiency and enzyme activation, despite the lack of information on the specific mechanisms through which the latter occurs), most of the specificity is determined by the catalytic domain, which is highly homologous between different AAAH (Erlandsen et al., 2002).

The *P. putida* AAAH investigated in this study is annotated as PheH at the NCBI database (www.ncbi.nlm.nih.gov/protein/24986222). In a genomic context, the *P. putida* phenylalanine hydroxylase gene (*phhA*, locus tag PP_4490) is part of a gene cluster that also includes *phhB* and *phhR* which encode a pterin-4-alpha-carbinolamine dehydratase (involved in regeneration of the pterin cofactor) and a sigma-54-dependent transcriptional regulator (operon activator), respectively. This genomic cluster seems to be conserved among other bacterial species (Jiménez et al., 2002).

In this study, AAAH activity was detected in the presence of Phe, but not with the other substrates tested (Trp and Tyr). AAAH activity with Phe was tested under different physical conditions (temperature, pH, $FeSO_4$ concentration) and the optimal temperature was determined to be around 25°C (Figure 12), even though different substrate concentrations as well as lower temperatures were not included in the experiment (due to equipment limitations), which should be explored in future experiments. Maximum AAAH activity was registered at pH 7.3 (Figure 13), and iron concentration around 5 µM (Figure 14). These biochemical characteristics seem to be shared with other bacterial PheH that have been characterized before, namely PheH from *C. psychrerythraea* (Leiros et al., 2007) and PheH from *C. violaceum* (Nakata, Yamauchi, & Fujisawa, 1979), which also exhibit maximum activity at 25°C and pH between 7.0 and 7.5. As for eukaryotic PheH, human PheH has a slightly higher temperature optimum of 30°C and a pH optimum between 6.5 and 7.5 (Ledley, Grenett, & Woo, 1987). These human PheH optimum biochemical conditions are similar for other human AAAH – TyrH (Szigetvari et al., 2019), TrpH 1 and TrpH 2 (McKinney, Knappskog, & Haavik, 2005). AAAH seem to share similar biochemical optimum activity conditions.

Due to unforeseen time and reagents limitations resulting from the constraints of the Covid-19 pandemic, different concentrations of the tetrahydrobiopterin co-factor (BH_4), and other related potential co-factors, were not tested as initially planned. When testing AAAH activity with different BH_4 concentrations, the BH_4 inner filter effect (IFE) must be considered. The IFE is a common problem in fluorescence spectroscopy measurements and, in the case of AAAH activity with phenylalanine measurement, in the presence of BH_4, the fluorescence signal intensity of tyrosine decreases with increasing BH_4 concentrations (Gersting et al., 2010).

To avoid this issue, BH_4 was included in the initial standard curve determination experiments, so it would not be a differentiating factor between independent experiments and readings.

Even though the results on the investigation of AAAH substrate promiscuity could not confirm what we initially hypothesized, and a more comprehensive enzyme characterization was not feasible in the available timeframe, this study is a good starting point for future research on this particular AAAH, which we know very little about. Also due to time and equipment limitations, other enzyme activity assays could not be performed. AAAH activity with tyrosine was not tested by TLC because, under the tested conditions, tyrosine, due to its higher polarity, does not migrate very efficiently on the silica plate with the mobile phase used for the other substrates (Figure 9), and the hydroxylated product (L-DOPA) migrates even less (not shown) since the additional hydroxyl group leads to an even stronger interaction with the polar stationary phase. Different TLC mobile phases with higher polarity were tested in previous experiments to achieve efficient separation of tyrosine and L-DOPA, but the results were inconclusive. Therefore, in this work, a colorimetric assay was used instead to successfully show that, under the tested conditions, no tyrosine hydroxylation is detected (Figure 11). Measurement of AAAH activity with a defined concentration of tryptophan using a fluorometric assay was also attempted but optimization of the assay conditions was not possible, and the results were inconclusive (data not shown). In addition to trying out other enzyme activity assays and techniques, different cofactors (other than tetrahydrobiopterin and iron) might be required for the substrate hydroxylation to occur, as the iron and cofactor binding is a critical step that leads to the suitable enzyme's conformational changes necessary for the substrate to bind to the active site (Erlandsen et al., 2002). So far, evidence points to BH_4 as the natural cofactor for most AAAH. However, natural occurrence of BH_4 is not very common in bacteria and some reports suggest that bacterial AAAH might use different pterin cofactors, such as tetrahydromonapterin (MH_4), which is the major form of pterin in *E. coli*, and experimental evidence has hinted about its cofactor function in some PheH (Lin et al., 2014). Furthermore, despite the apparent substrate preference for phenylalanine under our experimental settings, this *P. putida* AAAH may require higher tryptophan and/or tyrosine substrate concentrations for activity to be effectively detected. This has been effectively observed in other AAAH that, despite having higher catalytic efficiencies with their corresponding substrates, have different K_m values for different substrates i.e., for hydroxylation to occur they need greater concentrations of substrate to achieve V_{max}. Tryptophan hydroxylation by eukaryotic PheH has been reported before, with a K_m value 100-fold higher than that for phenylalanine (Teigen et al., 2007). Highly specific L-DOPA decarboxylase from *P. putida* KT2240 (which had been previously annotated as tyrosine

decarboxylase) showed minor activity towards 5-hydroxytryptophan, though with a catalytic efficiency (k_{cat}/K_m) 200-fold lower than for L-DOPA (Koyanagi et al., 2012). This L-DOPA decarboxylase residual activity with 5-HTP is of interest and must be further explored as it might be relevant in the hypothetical serotonin biosynthesis pathway suggested in this study, as it could act on the products of the *P. putida* AAAH whose characterization was initiated in this work.

Although a lot of questions remain unanswered, a lot of fascinating discoveries have been made in the last few years and, despite how challenging it might be, this is for sure a very promising field of research. Future efforts must focus on genomic analysis to provide a better understanding of the wild-type microbial genetics and possibly identify the key enzymes involved in serotonin and melatonin production, as well as their specificity and the optimal conditions under which they are most active. As more and more complete bacterial genome sequences become available and new genetic manipulation tools and other innovative techniques keep emerging, the search for new insights into the microbial physiology and ecology gets more accessible. Even though there is a large number of bacterial genes with no function prediction yet, and many homology-based predicted genes are sometimes mis annotated, it is often necessary to experimentally validate their function in order to better understand the complex microbial functional systems. Furthermore, detection and quantification of serotonin and melatonin is still very challenging as these compounds are produced in low quantities and the samples are often difficult to analyze, as. Thus, more effective techniques (or optimization of the currently used ones) that allow the simple and unambiguous detection and quantification of serotonin and melatonin (and their biosynthetic intermediates) in bacteria are essential (Danilovich, Alberto, & Juárez Tomás, 2021).

Considering the human gut microbiota as a possible source of serotonin and melatonin, further investigation on these indolamines' biosynthesis in bacteria might uncover key information on these metabolic pathways. It then becomes necessary to replicate previous discoveries and translate these into human conditions, as these new findings can potentially come to revolutionize current approaches to diseases related to serotonergic dysfunction and ideally open the path to the development of new bacteriotherapeutic strategies.

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Annex I

1. SDS-PAGE analysis

Resolving Gel 12%

Reagent	Quantity
Acrylamide/bis-Acrylamide, solution 29:1 (40%) (NZYTech)	1.688 mL
H_2O	2.445 mL
1,5 M Tris-HCl pH 8.8	1.41 mL
SDS 10% (w/v)	56.3 µL
Ammonium persulfate (APS) 10% (w/v)	56.3 µL
TEMED	7.5 µL

Stacking Gel 4%

Reagent	Quantity
Acrylamide/bis-Acrylamide, solution 29:1 (40%) (NZYTech)	179 µL
H_2O	1.21 mL
0,5 M Tris-HCl pH 6.8	470 µL
SDS 10% (w/v)	18.8 µL
Ammonium persulfate (APS) 10% (w/v)	18.8 µL
TEMED	3.8 µL

Running Buffer 10x

Reagent	Quantity (per liter)	Final concentration
Tris base	30.3 g	250 mM
Glycine	144 g	1.92 M
SDS	10 g	10%

2. Thin layer chromatography (TLC)

Ninhydrin solution

Reagent	Quantity
Ninhydrin	0.2 g
n-butanol	100 mL
Acetic acid	0,5 mL

The ninhydrin powder was dissolved in n-butanol. Acetic acid was added to the mixture and distilled water was added up to a final volume of 105 mL.