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Links between Serotonin Levels and Stress: Cortisol, *Candida a.*/Mycetes, Omega 3/6 Ratio and Dysbiosis (skatole/indoxyl sulfate) Role in Chronic Fatigue Syndrome (CFS) and Depression

Daniele Orlandoni[1*], Giuseppe Di Fede[1,2], Mauro Mantovani[1], Nava Consuelo[3], Marco Tomasi[4] and Paola Fusi[5]

[1]*Department of Medicine, Institute of Biological Medicine, Italy*
[2]*Department of Nutrigenomics and Pharmacogenomics, University of Pavia, Italy*
[3]*Department of Economics and Statistics, University of Torino, Italy*
[4]*Technical Director, IMBIO-Lab, Italy*
[5]*Department of Biotechnology and Biosciences, University of Bicocca Milano, Italy*

Abstract

Thanks to the large amount of evidence, a broader and more multidisciplinary vision of the intestine has emerged refers to the role that this anatomical structure plays in human body. A new sophisticated conception has arisen which has imposed a different approach in terms of investigating bowel importance and the repercussions that its functional deficit has towards other systems directly or indirectly related to it. It is, in fact, a complex structure interconnected with other systems (nervous, endocrine and immune) whose efficiency is strongly influenced by a condition of dysbiosis.

Intestinal microbiota attracts daily attention of a growing number of researchers and the data accumulated today allow us to highlight how dysbiosis plays a very important role in Irritable Bowel Syndrome (IBS), Inflamed Bowel Syndrome (IBD), Crohn's disease (CD) and even in Leaky Gut Syndrome (LGS) rather than Polycystic Ovary Syndrome (PCOS), food intolerance, diabetes, metabolic syndrome and fibromyalgia, cancer, etc.. However the composition of the microbiota is influenced diet, use/abuse of drugs, lifestyle and especially from stress and its reverberation on the Autonomous Nervous System (ANS), etc.

In our study we wanted to analyze how a condition of intestinal dysbiosis may be related to chronic fatigue syndrome (CFS) and depression through the exchange of information through the intestinal-brain axis (GBA).

We studied 33 subjects, 13 males and 20 females, who reported chronic fatigue syndrome or/and depression: We investigated their salivary cortisol levels, blood serotonin (5-hydroxytryptamine, 5-HT), omega 3/6 ratio, intestinal dysbiosis (calculated on the levels of urinary metabolites of tryptophan-TRP-: Indoxyl sulfate and skatole), and we looked for the presence of *Candida a.* or mycetes in the stool.

The data accumulated with this research show a correlation between the presence of *Candida a.*/miceti, indoxyl sulfate urine values beyond the physiological (characteristic of dysbiosis) and low 5-HT levels. In addition, data analysis showed that the EPA/DHA values also show pro-inflammatory levels in case of dysbiosis and low 5-HT levels.

The relationship, however, with cortisol levels requires further research although this study showed a statistically significant positive correlation between these values, measured at specific times, and 5-HT levels.

With this research we wanted to try to highlight the existing contact points, in some cases not so obvious, among these topics, contact points that, although they give us interesting indications, show the need to be further deepened by analyzing a larger amount of data.

Aim: We investigated the relationship between stress (evaluated through the measurement of salivary cortisol levels) and gastrointestinal efficiency measured as a function of intestinal fermentative and putrefactive dysbiosis, evaluating the levels of urinary indoxyl sulfate in the first case (a possible correlation with the presence of *Candida a.* or mycetes in the subjects feces was investigated), urinary skatole levels in the second one, in patients with chronic fatigue syndrome (SFC) and depression. In these patients we also have studied omega 3/6 ratio. Finally we have analyzed the impact that the alteration of all these parameters can have on the 5-HT levels.

This research attemps to highlight the contact points, in some cases not so obvious, among these topics, contact points that, although they give us interesting indications, show the need to be further deepened by analyzing a larger amount of data.

Keywords: Serotonin • Stress • Depression

***Address for Correspondence:** Daniele Orlandoni, Department of Medicine, Institute of Biological Medicine (IMBIO), Italy, Email: dott.orlandonidaniele@gmail.com*

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Introduction

All recent studies agree on the need to look at the intestine as a complex and interconnected structure with numerous systems starting with the nervous one in which the exchange of information and mutual influences is so strong that it has supported the need to identify a new axis: The gut-brain axis-GBA [1,2]. Thanks to this emerging vision, the microbiota now plays a very prominent role, so much so that the old description of the intestine as an "empty tube" now appears unreal in light of the impressive amount of bacteria that crowd the intestinal lumen: 1014 microorganisms belonging to about 400 different species [3,4] that literally crowd it with 9 million genes

[5], whose collaboration is essential to keep us healthy, for this reason the concept of microbiota understood as a virtual organ [6] or even forgotten organ [7] has gained ground. This is an interesting aspect of intestinal microbiology that has once again put researchers in difficulty because of the classic definition of organ intended as completely differentiated functional unit, and the microbiota does not fall into this classification, despite being involved in different metabolic activities through the synthesis of molecules involved in numerous metabolic functions [8].

The composition of the microbiota is strongly influenced by a multiplicity of factors, including the intake of fiber with the diet (Figure 1), an element that has changed over the years and since the so-called western diet began to spread, it has known a drastic reduction in favor of refined carbohydrates [9], at the same time the chronic inflammatory pathologies increased in general [10,11]. Impressive, in this sense, is the amount of works that link the alteration of the microbiota to environmental and oxidative stress [12], and to inflammatory diseases such as diabetes and colon-rectum cancer [13], IBS [14,15] and IBD [16], to CD [17,18] to LGS [19] in turn linked to asthma [20,21], to PCOS [22], fibromyalgia [23,24], autism [25,26], autoimmune diseases [27], with heart failure [28] and hypertension [29], we must not forget how nutrition [30], as well as the use/abuse of drugs and lifestyle, play a leading role in this sense [31].

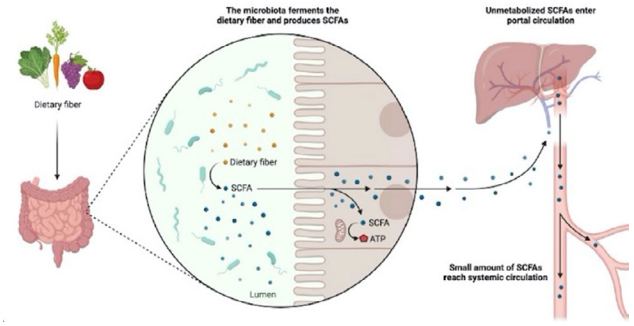

Figure 1. The fibers introduced with the diet are the essential nutritional substrate for the microbiota to produce short-chain fatty acids (SCFAs). The latter not only perform a fundamental trophic action at the level of the intestinal mucosa, but are also absorbed by passing into the circulation

However, there is another factor capable of profoundly influencing the composition of the microbiota: Stress [32] and its reverberation on the autonomous nervous system (ANS). This study arises from analysis of data relating to patients who have come to the Istitute of Biological Medicine in Milan (IMBIO, Italy) to find relief from their disorders (depression and CFS).

Our starting point was considering these pathologies as the expression of a condition of functional disregulation with respect to the reactivity of the biological system to stress (studied through analysis of the circadian levels of cortisol) as well as a functional imbalance that is realized through an inflammatory state (assessed through the omega ratio 3/6). The latter, in particular, linked to a condition of intestinal dysbiosis measured by urinary skatole (to evaluate putrefactive dysbiosis) and indoxyl sulfate levels (to evaluate fermentative dysbiosis) as well as in the analysis of the stool to determine the levels of *Candida a*. or mycetes among patients. Finally, all these parameters have been related to serum levels of 5-HT in order to evaluate any correlations.

In short, we wanted to analyze how a condition of intestinal dysbiosis can be related to CFS and depression by the exchange of information through the GBA measured through serum 5-HT levels, and how this exchange can be influenced by adrenal reactivity (intended as a measurement of salivary cortisol) and the omega 3/Omega 6 ratio because of their link with depression [33] and CFS [34].

Systemic approach to stress

The systemic approach applied to biology begins to make its way overwhelmingly thanks to the concept of System [35] and to Cybernetics [36], but already the experimentally measurable effects that emotions and stress produce on the organism in general and gastrointestinal efficiency in particular, had been tackled first with Cannon's work [37] and then with Selye's [38-40]. Selye, in fact, had shown that if an organism is subjected to a psychic stress, what we should expect is its manifestation in an organic key.

In short, attention is shifted from the single component of the system, to the relationships that the elements contract as a whole and how this interaction translates into a complex with characteristics different from the individual components of which it is made up (Figure 2).

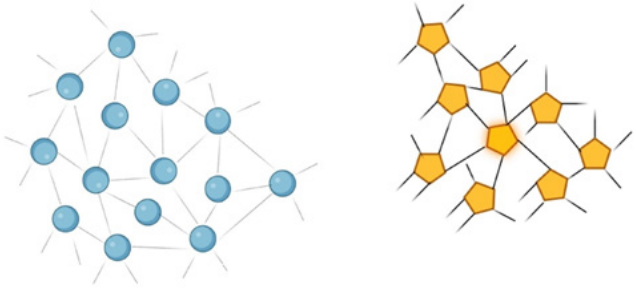

Figure 2. The study of the biological system passes from the analysis of its individual components (on the left in blue) to the evaluation of the network of relationships between them (in yellow on the right) and the resulting effects on the functioning of the system

This new point of view highlights another characteristic of living organisms clearly indicated by Bertalanffy: Being open systems. Basically an open system, that is every living being [41], is a system that exchanges matter with the surrounding environment, and to work in such a way as to produce and decompose structures thanks to its own constitutional organic resources; however, the information we receive from the outside disturbs the system, producing changes in its operation. The bacteria that crowd our intestine are a fundamental part of these interactions and from the type of relationship we are able to establish with the forgotten organ, it follows incoming and outgoing signals capable of influencing the functioning of distant structures such as the brain [42].

In relation to the effects that stress produces on our organism, the fundamental difference posed by Selye between eustress and distress should be noted. In both cases, in fact, the body responds in a non-specific and independent manner to the nature of the stressor but if the first is to be considered physiological, the second creates the conditions for the onset of the disease. From his studies it emerges that each phase of stress is accompanied by an increased energy demand, cortisol is a pivotal hormone in relation to stress reactivity: It is a molecule produced by the adrenal cortex [43,44] by activating the stress axis later to chronic stress of the organism.

Once the stressor, that is any internal or external factor capable of disturbing the biological balance, has been overcome, the organic system will move towards a physiological phase of recovery [45,46] of those previously mobilized resources; on the other hand, if the stress extends well beyond the resistance limit of the system, the body would enter a phase of exhaustion, hence the disease (Figure 3).

The regulation of cortisol levels follows a fine regulation controlled by the stress axis that relates the hypothalamus, pituitary and adrenal glands *via* the HPA axis [47], it should also be emphasized that the physiological levels of cortisol follow a pulsating trend in perfect agreement with our circadian rhythm [48], therefore a maximum peak is recorded between 06:00 and 08:00 in the morning and then see its values gradually decrease until they touch a minimum between 21:00 and 23:00 in order to predispose the organism to night rest.

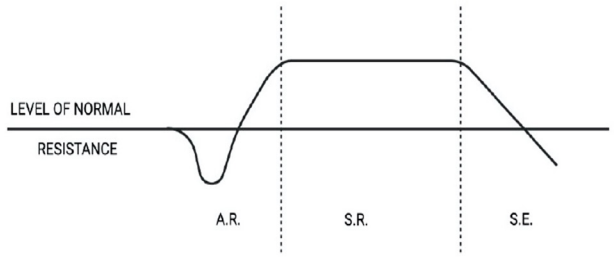

Figure 3: Physiological reactivity of the organism to stress in relation to time. After an initial phase of alarm (RA) governed by catecholamines, a second phase known as resistance (SR) which involves cortisol follows, if the duration of the stress continues. The latter can be maintained as long as the body is able to deal with it with adequate cortisol secretion based on its individual reactive capacity, in addition to this capacity the body will end up in a condition of adrenal exhaustion (S.E.)

Cortisolemia is now commonly measured in saliva [49] in the four physiologically significant ranges: 7:00, 13:00, 17:00 and 23:00; thanks to these measurements we are able to trace a cortisolemic profile of the person in order to evaluate his reactivity to stress (Figure 4).

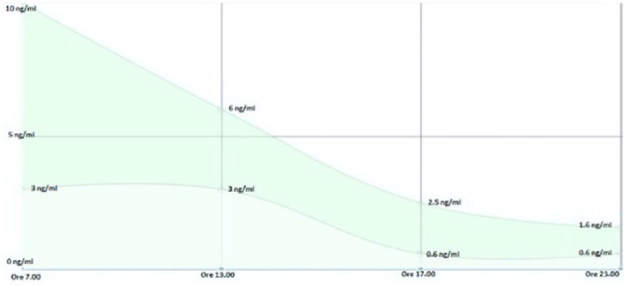

Figure 4: Physiological levels of cortisol according with our circadian rhythm. A maximum peak is recorded between 06:00 and 08:00 in the morning and then see its values gradually decrease until they touch a minimum between 21:00 and 23:00 in order to predispose the organism to night rest. (Ore=hours)

In conclusion, a subject subjected to a stressor will react through a first phase of alarm accompanied by the activation of the adrenal medulla and the consequent release of catecholamines, to then move on to the next phase of resistance with the activation of the adrenal cortex and consequent elevation of the levels of cortisolemia (Figure 3).

It is important to underline how the internal organs receive the simultaneous innervation of both systems (OS and PS), and that the effects of the activation of the two vegetative components on the aforementioned organs are antagonistic (Figure 5). The two systems work together to allow the organism to tend to the maintenance of a condition of physiological equilibrium or homeostasis [50], a vision that has undergone a profound re-evaluation thanks to the work of Bertalanffy [35] on systems, with the introduction of the concept of steady state (Figure 6).

It is a more complex conception of equilibrium of the biological system, which includes the idea of equifinality. Open biological systems tend towards a time-independent state: The steady state, an equilibrium, without ever reaching it fully (and that is why they are capable of producing work). The tendency towards a steady state is independent of the initial conditions of the system, therefore unlike what occurs in closed systems, the final state is achieved thanks to equifinality, although starting from very different initial conditions and after the process has been perturbed in several ways. These concepts are applicable in many sectors of biology [51-53] in the human body, in fact, many tissues remain in a stationary state while their cells continuously go into apoptosis, or are in any case eliminated, to be continuously replaced.

In the light of what has been illustrated, we can affirm that both if the organism is in a situation of sympathotonic hyper-reactivity (valid for both acute and chronic), and hyper-parasympatheticotonia (with the mobilization of the mediators of inflammation), the biological system will, however, tend towards its stationary equilibrium over time. Obviously, all this is achieved as long as the system is able to restore balance from the autonomic and biochemical point of view; but what happens if the ANS finds itself stuck in one phase rather than in its opposite phase?

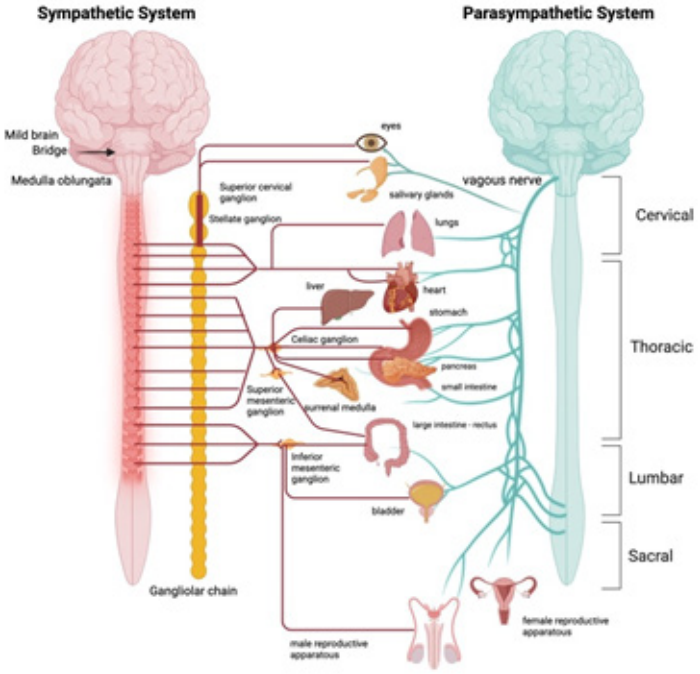

Figure 5: Anatomy of the autonomic nervous system (ANS): in red the fibers of sympathetic system, in green those of the parasympathetic

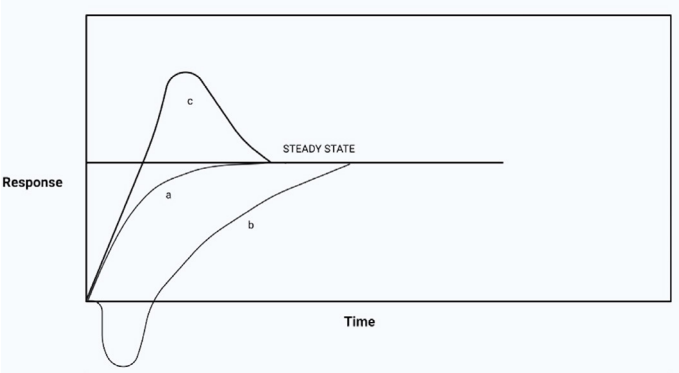

Figure 6. Any system, in accordance with von Bertalanffy's general systems theory, regardless of the starting conditions (a, b or c), will always tend towards a steady state

In case it were unable to interrupt the activation of the OS, if the stressor (physical or emotional) continues over time, the system will go through a first acute phase (alarm) which is characterized by the presence of catecholamines, followed by the subsequent chronic phase (resistance) that will last as long as the organic resources will allow, in addition to this point the biological system will pass the point of no return, delivering itself to the disease phase understood as a biological standstill that allows the body to recover the resources already committed to the response to stress.

In case it was unable to interrupt the activation of the PS, the biological system is blocked in a phase predominantly dominated by the PS and characterized by intense vagotonia caused by insufficient vagolytic action (condition of chronic inflammation).

CSF and depression

CFS is a severely debilitating condition in which patients complain of a varied range of symptoms including constant fatigue, immune alterations as well as musculoskeletal pain [54], with a very similar picture to IBS [55]. Patients suffering from CFS are simultaneously suffering from neurological impairment, cognitive dysfunction [56,57] and gastrointestinal disorders.

A significant increase in facultative anaerobic bacteria within the microbiota of these patients is clearly recorded, as well as a condition of acidosis characterized by a high level of lactic acid [58]. We know that lactic acidosis is a condition accompanied by neurological weakening that includes ataxia, dysarthria, weakness and difficulty concentrating [59]. The condition of acidosis may be due to an excess of carbohydrate fermentation [60], an increase in *E.coli* and the proliferation of facultative anaerobic bacteria [58]. As I have already had the opportunity to illustrate, it is precisely when this type of bacteria begins to make their way, to the detriment of obligate anaerobes, that the picture of intestinal dysbiosis begins to take shape.

Depression is a widespread pathology on a global scale, a condition which, without adequate treatment, is characterized by episodes which can last from 4 to 12 months, characterized by a state of mind of profound despondency which characterizes the whole day and which it becomes more and more persistent as time passes. It is a condition accompanied by acute mental suffering, characterized by the inability to perceive pleasant feelings (anhedonia) and by a loss of interest in the world and the things in life for which one previously felt satisfaction. Its diagnosis requires at least three of the symptoms listed below: Reduced appetite associated with weight loss (on other occasions hyperphagia and weight gain), weakness, decreased libido, restlessness, alteration of the sleep-wake cycle with hypersomnia or insomnia with awakening early or with frequent awakenings during the night, psychomotor retardation, difficulty concentrating, indecisiveness, pessimism, thoughts of death and suicide, sense of guilt, perception of personal uselessness. Other symptoms may also be present even if considered not as important in terms of diagnosis: Constipation, decreased salivation and exacerbation of symptoms especially in the morning. It is interesting to analyze the spread of anxiety and depression in the world through a 2013 work [61] from the study of the data contained, it appears clear that the evaluation of the impact of symptoms such as anxiety and depression is largely underestimated at a global level since in a large part of the planet no reliable data or precise statistics are available (in many cases they are not even filled in).

Depression, glucocorticoid resistance and dysbiosis

The correlation between depression and the microbiota has been clearly highlighted [62,63], as is the correlation between dysbiosis and stress [64], or dysbiosis and anxiety [65].

Furthermore depressed patients often present strong anxiety crises which apparently should not be highlighted since in depression there is a parasympathetic, i.e. vagal, dominant activation which characterizes the typical traits of this pathology; in reality anxiety represents a desperate, and strategically coherent, attempt by the organism to interrupt the parasympathetic prevalence through a sudden acceleration of the orthosympathetic also tending to limit the inflammatory process that accompanies, or generates, the depression itself [66].

Moreover, when analyzing the consequences that occur following the activation of the stress axis, we must always consider its essential component as the starting point for any reflection: The hypothalamic-pituitary-adrenal axis (HPA) axis. This is the key aspect that intervenes in regulating the response to stress; remembering how its hyperactivity or lack of regulation of negative feedback inherent in a reduced response to glucocorticoids if not even in resistance to the latter, represents a pattern widely present in depression [67,68].

Cytokines produce different effects on the HPA axis: The administration of pro-inflammatory ones exerts an influence on the latter through the stimulation of the expression and release of CHR [69,70]. The increase in cortisol levels following stimulation of the HPA axis is a physiological and functional response to the need to put a brake on the inflammatory process, foreseen and triggered at the end of the reactive phase of response to stress, through the inhibition of the release of other pro-inflammatory agents through its action on cells of the immune system [71]. In this sense it is important to underline how in depressed patients, cortisol seems to lose its ability to intervene on the latter as a limiting factor with respect to the inflammatory process itself, on the other hand it still manages to prevent a further release of pro-inflammatory cytokines [72] in fact, administering INF-γ to healthy subjects results in an increase in cortisol values [71].

Once again, the mechanism through which stress is able to produce an inflammatory response involves catecholamines and the HPA axis [72] once again it must be underlined that even psychosocial stress which includes interpersonal conflicts as well as social isolation rather than traumatic events are capable of causing the release of CRH and catecholamines first, then cortisol. The interaction of the former with the adrenergic receptors would lead to an increase in NFkB-DNA binding with consequent release of inflammatory mediators [73] these mediators, cytokines, can reach the brain and thanks to their action on microglia, activate the inflammatory cascade with consequent neuro-inflammation. Therefore, the CRH stimulated by cytokines would end up activating the HPA axis in order to stem the inflammatory response, however this mechanism would be missing in pathological conditions such as depression or chronic stress: In these cases cortisol is no longer able to produce this control and to prevent a further release of cortisol, adding adrenal stress to chronic adrenal stress. This is a condition known as glucocorticoid resistance [74].

The activation of the stress axis involves a remodeling of the microbiota, a condition of dysbiosis which indicates the breakdown of the delicate balance that characterizes the microbiota useful for the correct functioning of a healthy intestine; altering the bacterial presence in this sense leads to a significant series of effects on various levels, including the digestive one, in particular the intestinal mucosa is exposed to the presence of specific substances, indoles [75].

The intestinal mucosa, nutrition, bacteria and inflammation

The intestinal mucosa represents our real interface with the surrounding world since on average around 60 tons of food transit throughout our life cycle [76]; this is an extremely relevant detail since it is food the fundamental element to determine the well-being of the individual in a more general perspective, the composition and activity of the microbiota in a more particular one [77-81] however framed in a long-term perspective. We must always remember that it is thanks to a correct microbiota that we are able to effectively stimulate our immune system, produce vitamins (of group B and K), activate the motility and function of the gastrointestinal tract, perform digestive and absorption processes correctly, to prevent colonization and the consequent pathogenicity of any pathogens, the production of short-chain fatty acids (Figure 1) SCFAs [82,83], in particular butyric acid a fundamental molecule for the trophism of the intestinal epithelium as well as performing a very important anti-inflammatory function [30]. Finally, we must remember that the products of bacterial metabolism represent 40% of the molecules in circulation [8].

As regards the composition of the microbiota, it should be emphasized that it is an extremely delicate balance, subject to many variables capable of profoundly influencing its fate, in particular in relation to the effect that antibiotic therapy shows on the intestinal bacterial composition [64], even if limited to a single dose [84] it is able to produce a very substantial increase in *Enterobacteriaceae*. An alteration of this balance leads to the onset of particularly dangerous conditions from an inflammatory point of view, both local and systemic, an alteration that manifests itself, for example, with the decrease in the presence of *Faecalibacterium prausnitzii*, an obligate anaerobic microorganism used as a marker of intestinal eubiosis, and the simultaneous increase in the proliferation of a facultative anaerobic bacteria such as *E.coli* and *Enterobacteriaceae* in general, rather than real aerobic bacteria; in this way the presence of bacteria belonging to these last two groups is a useful indicator for determining a condition of intestinal dysbiosis [85]. It is interesting to note that the increase in facultative anaerobic bacteria is recorded not only in the case of inflammation-induced dysbiosis, but also following treatment with antibiotics [86].

As just mentioned, the mucosa is continuously exposed to bacterial products-endotoxins [87]; hydrogen sulfide-H_2S [88]; phenols, ammonia and indoles-which can produce harmful effects both on the mucosa itself and on our health [75]. The presence of many of these toxic metabolites depends on the type of fermentation that develops in the intestine, which in turn depends on the type of bacteria that the intestine itself receives, as well as the substrates available for fermentation.

Dysbiosis

The considerations made so far clearly highlight how the changes in the Western lifestyle, which have occurred so rapidly in recent decades, characterized by a strong increase in the dietary consumption of fats and sugar, have altered the composition and metabolic activity of the human microbiota [89]. On the other hand a diet rich in protein, and low in fiber, is able to determine the formation of highly toxic products with decidedly pro-inflammatory characteristics. Already in the early 20th century, Metchnikoff postulated how diseases and biological aging itself were generated by the putrefaction of proteins in the intestine and how the intake of probiotics was recommended to favor detoxification and support a correct bacterial composition [90,91]. This is the toxicological theory of the intestine which soon turned into the term dysbiosis coined by Metchnikoff himself [92] to describe a pathogenic bacterial alteration in the intestine; while others later referred to dysbiosis to indicate: "... qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution." At this point, dysbiosis must be considered as a state in which the microbiota produces dangerous and harmful effects through:

- Qualitative and quantitative alterations of the microbiota itself;
- Changes in the metabolic activity of intestinal bacteria;
- Variations of their local distribution.

Starting from these assumptions, the proliferation of potentially pathogenic bacteria in the intestine is the cause of the release of numerous toxic products, previously mentioned, which end up playing a leading role in numerous chronic and degenerative diseases. In short, dysbiosis opens the door to possible interventions of great prospect in numerous chronic degenerative pathologies on an inflammatory basis.

Candida a. and mycetes

Different species of Candida can be present as commensals, however it is *Candida a*. the most common, and the most studied, causative agent of disease in humans. *Candida a*. is an opportunistic commensal and a natural saprophyte whose primary site of colonization is the Gastro-Intestinal tract (GI) which runs from the mouth to the rectum, and vagina [93].

The risk elements linked to the proliferation of *Candida a*. in the GI tract are to be identified in factors such as medically induced immunosuppression (corticosteroids or chemotherapy-induced neutropenia), or the use of broad-spectrum antibiotics [94] in some cases associated with the alteration of the microbiota capable of causing the translocation of *Candida a*. through the intestinal mucosa [95,96].

Mucosal infections, characterised by fungal colonisation (i.e. overgrowth) associated with an inflammatory host response, are extremely common and can have a major impact upon the quality of life for many individuals; most women of reproductive age (75%) will experience at least one episode of Vulvo Vaginalis Candidiasis (VVC) in their lifetime, and up to 9% suffer from recurrent VVC, as defined by multiple episodes of vaginitis per annum [97,98].

Moreover, colonization by *Candida a*. It is capable of producing numerous effects by stimulation of specific toll-like-receptors (TLRs) causing the expression of inflammatory cytokines [99], *Candida a*. also produces H_2S capable of carrying out an antioxidant action towards the oxidative response implemented by the innate immune system [100].

It is also important to underline how an overgowth of *Candida a*. is able to exert effects on the CNS through the production of acetaldehyde (ACD) comparable to those of alcohol consumption [101]. But the most important aspect that suggested us to evaluate the presence of *Candida a*. in the intestines of the patients involved in the study, is the similarity between the symptoms of a *Candida a*. infection a. and CFS [102,103].

Starting from these assumptions we wanted to go further by also wanting to evaluate the presence of mycetes in the feces of patients in order to be able to find a possible relationship between these and intestinal fermentation expressed through urinary indoxyl sulfate levels. In the end, we wanted to investigate whether intestinal fermentation, supported by an overgrowth of *Candida a*. or mycetes, was related to the decreased 5-HT levels in the patients included in the study.

Tryptophan, serotonin, melatonin and intestines

Brain 5-HT levels are closely related to the intake of TRP in the diet. The enzyme most involved in its metabolism is TRP-hydroxylase which, to be activated, requires adequate amounts of folic acid, vitamin B6 and magnesium. TRP is an essential amino acid capable of crossing the blood-brain barrier (BBB) and reaching the brain; this is a very relevant detail since it is in the brain that its transformation into 5-HT or melatonin takes place, a process linked to the light/dark, sleep/wake cycle. The mechanism illustrated in Figure 7 is the one which leads to the production of 5-HT and melatonin in the brain under the control of the pineal thanks to the electrical activity of the suprachiasmatic nucleus [104].

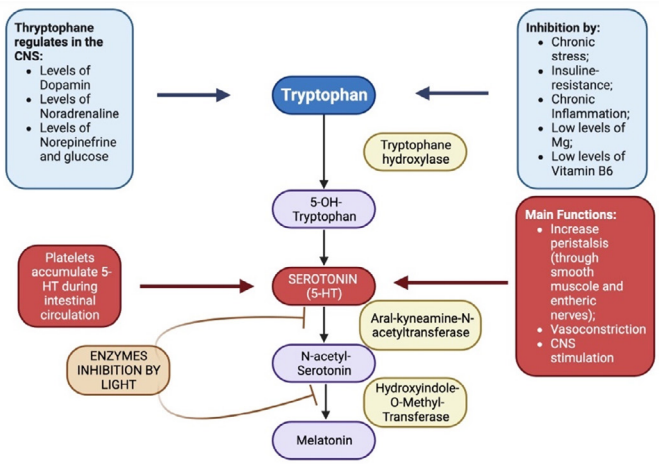

Figure 7: Biosynthesis of serotonin from tryptophan.

But these are not the only variables with respect to the conditions that can influence the levels of 5-HT: Subjects who face a condition of psycho-physical stress or who complain of emotional instability, record an increased consumption of TRP [105] measured as a decrease in its plasma levels. As proof of this evidence it has been shown [106] that if the availability of TRP is reduced to healthy subjects, or in people who have recovered from depression, there is a decrease in mood and the reappearance of depressive symptoms which in males presented itself as anger associated with aggression and impulsive attitude, while in females it manifests itself as closure in itself.

It should also be emphasized that an excessive proliferation of E. coli is also capable of producing a decrease in 5-HT levels since it uses TRP to produce indole and after hepatic metabolism it is eliminated as indoxyl sulfate in the urine [107]; this leads to a drastic decrease in intestinal 5-HT levels which ends up reflecting on mood by virtue of the gut-brain axis [1,2].

However, there is a crucial aspect that draws our attention to the relationship between the intestine and serotonin, which consists in the fact that 95% of the 5-HT in our body is synthesized in the intestine, while only 1% sees the light in the brain.

It becomes clear how important it is to ensure the body has a balanced diet in terms of ratios between carbohydrates and proteins as well as fiber intake, in relation to the stress load it is facing; Furthermore, it is essential to maintain a correct digestive phase, in order to avoid fermentative or putrefactive processes that could compromise the metabolism of TRP. In the latter case, it must be remembered that an organism subjected to both acute and chronic stress (the former characterized by the production of catecholamines while the latter by an increase in cortisol levels) is accompanied by a decrease in vagal activation, the same one that characterizes the digestive phase as a whole. In other words, stress, whether acute or chronic, creates the conditions to prevent the digestive tract from carrying out its duty, laying the foundations for the subsequent fermentative or putrefactive processes that represent, through the selection of the suitable microbiota from a functional point of view. (such as candida or fungi), an adaptive response that allows, although improperly, to exercise a digestive/assimilation function alternative to the physiological one.

Tryptophan shunt

I have already mentioned how chronic stress is accompanied by high levels of cortisol, i now want to highlight another important aspect of this condition and that is linked to the TRP shunt. Towards the end of the sixties, important research was published in the Lancet [108] regarding the metabolism of this amino acid, the alteration of which was indicated as the main cause of depression since it is the dose-limiting substance production of 5-HT given that less than 5% of the metabolism of this amino acid follows this pathway, while 95% is metabolised in the kynureine pathway [109,110]. Also in the sixties, another research focused on the role of TRP [111]: Essentially, it was hypothesized that the amino acid would not be used for the synthesis of 5-HT but diverted towards production of kynureine (KYN); it would be a mechanism activated by stress hormones through TRP 2,3-dioxyoxygenase (TDO), by the reduced availability of TRP following the synthesis of KYN thanks to the TRP itself and above all by the increase in cortisol synthesis by the adrenals as a response to the decreased inhibitory effect produced by 5-HT on the amygdala.

Furthermore, KYN produces an important effect: It is able to inhibit the transport of TRP across the BBB and to stimulate the activity of indoleamine 2,3-dioxygenase (IDO) which is in turn activated by pro-inflammatory cytokines and, together to TDO, limiting enzyme of the TRP-KYN pathway; KYN in animal models is also capable of generating anxiety [109,112].

It should also be underlined that TDO is activated precisely by TRP [109] and given that KYN inhibits transport across the blood-brain barrier and competes for the cellular up-take of TRP and causes inhibition of the substrate on TRP-hydroxylase, if there is an excessive increase in TRP itself, a decrease in 5-HT synthesis can be generated [113].

The link between low levels of 5-HT and depression has already been widely demonstrated by numerous studies, just as TRP depletion is able to experimentally produce a depressive picture in healthy subjects [108]. Once the conflict has been overcome, the system will physiologically tend towards a stationary state (Figure 6) according to the principle of equifinality [35], first passing through a necessary recovery phase.

In the second case the conflict continues without finding a solution, the person will therefore find himself in a condition of chronic stress, with all the consequences illustrated previously, which will force him to put his organism to the test, requiring the exploitation of huge organic resources ; as long as the latter are available, not without discomfort, the person will be able to keep the stress axis active, but what happens when the limit is exceeded, when the demand for resources exceed the quota actually available? The person will enter a phase of adrenal exhaustion with a clinical picture characterized by chronic fatigue and low mood, a generalized inflammatory state so profound that it does not allow the biological system (again according to Bertalannfy's principle of tendency to steady state) to return naturally towards its steady state; furthermore, it is now accepted that inflammation is a process that can lead both to the onset of a physical pathology and to a neuropsychiatric pathology such as depression [74]. In the latter, in fact, the same inflammatory markers are expressed [114].

In short, until the stressful conflict is resolved and the natural balance between orthosympathetic and parasympathetic activation is re-established through a physiological inflammatory phase of recovery of the resources used during the resistance phase, the adrenals will continue to be stimulated: The more the condition of resistance is prolonged, the more prolonged the subsequent and, I would like to point out, physiological inflammatory phase will be. A profound inflammatory response resulting from prolonged stress can, however, precipitate an equally profound inflammatory phase which will tend to become chronic through the expression of specific mediators which have already been associated with depression [115,116].

Gut-brain Axis (GBA), stress and microbiota

I have already mentioned how compromising the delicate balance of our microbiota leads to a condition of dysbiosis, a condition that is evidenced by a deficit of intestinal function and mood disorders, both connected with the disruption of the GBA [117,118] as is shown in Figure 8.

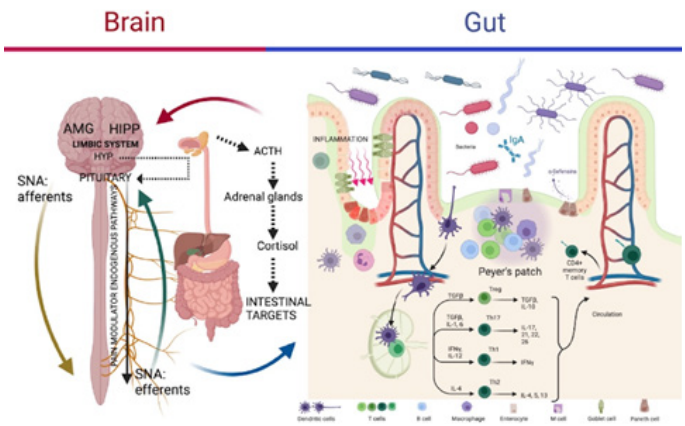

Figure 8: The left side of the figure (Brain) shows the information exchange routes that reach the gastrointestinal system from the brain. It should be emphasized that this is a two-way path and that the information that travels upwards from the bottom is greater than what is recorded from the top to down. On the right side (Gut) we can observe how the presence of pathogens in the intestinal lumen is able to activate inflammatory signals, which mediators are involved and how the immune

In a work published by the Journal of Clinical Psychiatry [119], the researchers divided mice into two groups: One control, while the other brought together mice previously infected in the intestine with Campylobacter jejuni, the latter group showed neither signs of infection or circulating inflammatory cytokines, however they were clearly more anxious than the controls. At this point the question was how communication between the intestine and the brain is achieved since no circulating mediators linked to infection and inflammation is measured. The answer to this question must be sought in the particular anatomy of the gastrointestinal system in general, in the intestine in particular, because the signal from the periphery to the center is transported by the viscero-sensory nerves that innervate the intestine, they are pathways that carry their signals to the brain, signals coming from the presence of bacteria in the intestinal lumen, which influence its functioning. Vagal innervation, in particular, develops a close relationship with the immune cells of the submucosa [120,121], projecting its nerve endings up to the mucosa. The signals coming from the bacterial presence are able to influence brain functions through their transport through both the vagus nerve and the fibers of the spinal visceral sensory nerves [122].

Several studies [123,124] bring to our attention how the viscerosensory nerves provide a complex contribution to the dysfunctional response of the intestine associated with stress; in particular, the stimulation of the vagal intestinal component would produce anxious-depressive states in many patients. Thanks to numerous researches, data has been collected to support the thesis according to which regions of the brain involved in emotions are associated with autonomous viscerosensory signals [125-128].

A very interesting data is that the risk of IBS increases 10 times when the subject reports a previous gastroenteritis [129], in many cases it is the consequence of a gastroenteritis acute [130], a condition called post-infectious IBS; it should be noted that subjects in whom the diagnosis of IBS is more recent, show anxiety rather than depression, while when IBS is chronic or resistant to treatments, patients present anxiety and depression together; finally, IBS itself is often accompanied by chronic fatigue syndrome [131,132].

What has been said so far allows us to highlight the clear association of a specific symptomatology, in particular CFS as well as anxiety and depression, with gastrointestinal disorders, especially as a consequence of a state of inflammation or infection [133-136].

Finally, it is known how a multiplicity of stressors is able to modify the composition and the overall biomass of the intestinal microbiota and this independently of its duration over time. In fact, it has been shown that exposing a subject to a social stress lasting only two hours, so as to activate the response of the HPA axis, is sufficient to determine a remodeling in the bacterial profile and in the relationships existing between the different phyla [137], including the genus Lactobacillus [138-142].

To conclude, it must always be remembered that the ENS represents the privileged target of bacterial metabolites; one of the most significant products in this sense, as I have already mentioned, are the SCFAs (acetic, butyric, propionic acid), substances capable of producing a stimulation of sympathetic fibers [143], the release of 5-HT from the intestinal mucosa [144], as well as influencing the memorization and learning processes [145,146].

Omega-3/Omega-6 ratio

Fatty acids perform structural, energy and metabolic functions in living organisms. The fatty acids of the Omega-3 and Omega-6 series represent a group of substances essential for our organism for the production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the case of the fatty acids belonging to the first series called PUFA altogether, arachidonic acid (AA) as regards the second.

From the study of Figure 9, it can be easily deduced that the quality of the diet will therefore be decisive in establishing the plasma ratios of the fats of the two series: An unbalanced diet towards foods rich in Omega-6 will support a physiological tendency to amplify inflammatory processes; on the contrary, a prevalence of Omega-3 will produce the opposite effect. It is currently calculated that in the Western diet the Omega-6/Omega-3 ratio is equal to 20-25: 1 [147], and that this imbalance affects 70% of the American population [148] therefore clearly pro-inflammatory.

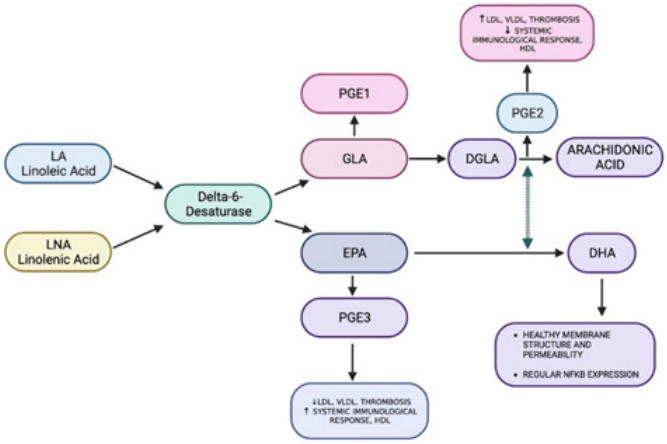

Figure 9: From the study of the biochemical path involving omega 3/6 fatty acids it is clear that a diet that favors foods rich in omega 6 leads to the production of arachidonic acid and a consequent inflammatory state; conversely, a diet rich in omega 3 shifts the balance towards a greater presence of EPA and DHA with a consequent anti-inflammatory effect

Because of their ability to carry out a natural anti-inflammatory action [149], Omega-3s are studied and used in the therapy and prevention of much pathology: Neuropsychiatric [150], cardiovascular, in gastrointestinal cancer, etc. [151]. The key role played by these substances is particularly important to us since their link has also been demonstrated in depression [152].

Data and Methods

***Candida a.* and Fungi:** Biological samples are collected and carried with specific transport medium (Amies Swab). Microorganisms are grown with standardized culture medium: Enriched media and differential media.

Used media are

Chromagar Candida: Chromogen medium useful for the growth and semi-

quantitative, immediate identification from mixed yeasts cultures (Candida spp.)

Saboraud agar: A selective medium useful for the isolation of fungi and yeasts from sanitary and clinical samples. So inoculated media have been incubated for 48 hours at 37°C in the presence of air. The purpose of the sowing of the samples is to obtain cultures from which it is possible to produce pure colonies of known pathogens, in order to proceed with the certain reporting of the microorganism found.

Salivary cortisol: The cortisol values were measured on a saliva sample collected at four different times of the day (7AM-1 PM-5 PM-11 PM), on an empty stomach and away from the intake of any food or drink for at least 1 hour, using as a means of collecting salivettes®.

Enzyme immunoassay method: The Diametra Cortisol Saliva ELISA kit was used; the kit includes a plate pre-treated with the anti-cortisol antibody. It works thanks to the competition between cortisol and horseradish peroxidase (hrp): If the saliva sample contains little cortisol, the hrp binds to the antibody giving a blue colour to the sample; the more cortisol is present, the less dark the sample is. The addition of the so-called STOP solution stops the reaction between cortisol, antibody and hrp by changing the various samples to yellow of different intensity. Spectrophotometric reading at 450nm follows. By comparing the samples with the known values of the calibration curve (0 pg/ml–0.5 pg/ml–1 pg/ml–5 pg/ml–10 pg/ml–20 pg/ml–100 pg/ml) we can trace the concentration of the tested samples. The reference values vary within the day.

Dysbiosis and serotonin: The quantification of skatole is done in HPLC (High Performance Liquid Chromatography): After having purified the sample (5 ml of morning urine) by filtration with 0.4 µm filter syringes, it is analysed with a RID (refraction index detector) placed at the end of a selection column (NH4) containing resin suitable for separating the TRP products. The sample reading is compared with a calibration straight line prepared previously by reading known skatole concentrations (10 µg/l–20 µg/l–40 µg/l) and the sample content is then determined. The procedure is then repeated for the indoxyl sulfate with the difference of the calibration line (10 mg/l–20 mg/l–40 mg/l).

5-HT is also dosed by HPLC, in this case, however, serum (1 ml) is used as a biological sample, which is purified by centrifugation and subsequent filtration in 0.4 µm filter syringes. A 75% acetonitrile eluent solution diluted in water is used for all three analyses.

The concentration of 5-HT is read thanks to an optical UV reader placed at the end of a column (C4) containing resin capable of separating the proteins, it is then compared with a calibration line prepared previously by reading known concentrations of 5-HT (100 µg/l–150 µg/l–200 µg/l–250 µg/l); the concentration of the sample under examination is then determined.

EPA and DHA: Fatty acid extraction: 10 µl of human serum were mixed with 10 ml of Heptadecanoic acid 50 mM as Internal Standard and 10 ml of CH_3CN (acetonitrile); therefore, 30 ml of Perchloric acid was added to deproteinize, and 800 ml of chloroform, 100 ml of methanol, 100 ml of n-heptane and 100 ml of H_2O were added to extract the fatty acids.

After the solution was centrifuged at 3000 rcf for 5 min, the organic layer (400 ml) was transferred to a dark tube where the solvent evaporated. A residue is collected under reduced pressure using a centrifuge concentrator, VC-36N (TAITEC Co., Ltd., Saitama, Japan) without heating for 20 min.

The dry residue was dissolved in 50 mM DMF and mixed with 50 ml of 2 mM DBD-ED in CH_3CN, 50 ml of 140 mM TPP in CH_3CN and 50 ml of 140 mM DPDS in CH_3CN to derivatize the fatty acids. After waiting at room temperature for 120 min, an aliquot of the solution was diluted 50 times with CH_3CN–H_2O (7:3). The diluted solution was filtered and 10 mL was analysed with HPLC.

HPLC: The HPLC system used consisted of a pump, a column oven, a degasser and a fluorescence detector (1220 INFINITY II LC, Agilent). A CD-C18 column was used in a column oven at 40°C and the flow rate was constantly maintained at 1.0 mL/min. The excitation and emission wavelengths of the fluorescence detector were set to 450 nm and 560 nm, respectively. The mobile phases A, B and C used in the study were 0.1% TFA/CH_3CN, 0.1% TFA/H_2O, and 0.1% TFA/MeOH, respectively. The time schedules for the mobile phase were as follows: 0-25.00 min A% ¼ 60-70, B% ¼ 30-20 (linear gradient) and C% ¼ 10; 25.01 min-40.00 min A% ¼ 70, B% ¼ 20 and C% ¼ 10 (isocratic); 40.01-55.00 min A% ¼ 70-77, B% ¼ 20-13 (linear gradient) and C% ¼ 10; 55.01 min-65.00 min A% ¼ 77, B% ¼ 13 and C% ¼ 10 (isocratic) and 65.01 min-84.50 min A% ¼ 60, B% ¼ 30 and C% ¼ 10 (initialization). The resulting chromatogram was analysed using OpenLab software (Agilent). Calibration curves for ALA, PLA, AA, LA and OA were constructed in concentrations of 1 mM-50 mM, 1 mM-200 mM, 10 mM-200 mM, 25 mM-800 mM and 25 mM-800 mM, respectively. The peak area ratio of the Internal Standard was then plotted on each concentration (n¼ 4).

Results and Discussion

For the research, 33 subjects were recruited, 13 males between the ages of 26 and 77, and 20 females between the ages of 25 and 69. Each of them reported symptoms largely similar in many respects: Low mood, abdominal swelling, fatigue, sleep disorders, anxiety, and weight gain.

To highlight the link between HPA and dysbiosis [153,154] and between the latter and inflammation, the levels of indoxyl sulfate and skatole were evaluated and indicated urinary (skatole as a measure of putrefactive dysbiosis and indoxyl sulfate as a measure of fermentative dysbiosis), the possible presence of candida or mycetes in the faeces, together with the plasma 5-HT levels, and the ratio Omega-3/Omega-6 still evaluated in plasma.

Cortisolemia levels were also measured, but not in all subjects, assessed in the four physiologically significant moments, namely at 7 am and at 1 pm, 5 pm, and 11 pm.

Figure 10 shows the graph of the correlations between the main numerical variables considered in this research: The larger the colored circle, the stronger the correlation (the black color indicates a strong positive correlation, while the white ones a negative type). The same correlations are shown in the right table but expressed in numerical terms. To this end, it should be remembered that the calculated linear correlation can assume values between -1 and 1.

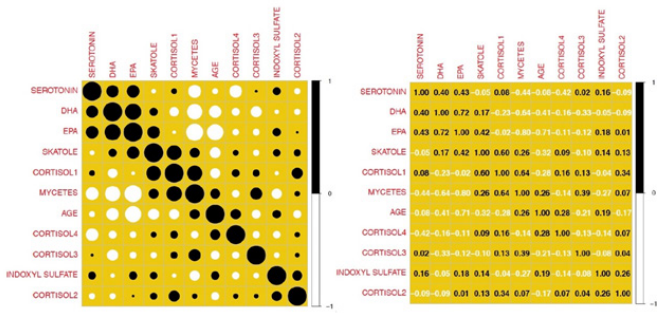

Figure 10: Correlations between the main numerical variables considered in this research: the larger the colored circle, showed in the left table, the stronger the correlation (the black color indicates a strong positive correlation, while the white ones a negative type). The same correlations are shown in the right table but expressed in numerical terms

A correlation, in absolute value, equal to 1 indicates a strong linear dependence, while values close to zero reveal the absence of a linear link between the variables considered. From the analysis of the above values it can be seen that a positive correlation is highlighted between the levels of 5-HT and those of EPA and DHA, while a negative one emerges between these last two parameters and the number of fungi found.

Another interesting aspect that I want to emphasize is how the two fatty acids show a strong negative correlation (EPA to an even greater extent than DHA) with the age of the subjects considered in the study. Finally, 5-HT is negatively correlated with the number of fungi and with Cortisol-4 (measured at 11 pm). Cortisol-1 (measured at 7), on the other hand, shows a modest inversely proportional link with age, while Cortisol-4 (measured at 11pm) exhibits an equally modest but directly proportional link.

In order to identify the behavior of the numerical variables considered also with the variation of some observed parameters/variables, the following boxplots were then analyzed. In Figure 11 the levels of 5-HT, skatole and indoxyl sulfate are analyzed in relation to the presence or absence of *Candida a*.; in the first case (Figure 11) it is evident that the plasma levels of 5-HT are on average higher in the 3rd and 4th quartile than in patients who do not present *Candida a*.. In relation to skatole (Figure 11) this evidence is not recorded since its levels appear substantially identical between the group of patients with *Candida a*nd those without.

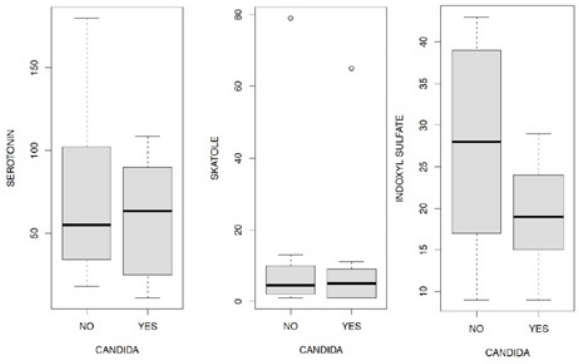

Figure 11. Levels of serotonin, indoxyl sulfate and skatole analyzed in relation to the presence or absence of *Candida a*.. Figure 11.1 shows that the plasma levels of serotonin are on average higher in the 3rd and 4th quartile than in patients who do not present *Candida a*.. In Figure 11.2 we can observe how this evidence is not recorded since skatole levels appear substantially identical between the group of patients with *Candida a*nd those without. At last, Figure 11.3 display how the levels of indoxyl sulfate, expressed both in the 2nd and in the 3rd and 4th quartiles, are clearly higher in the case in which it is excluding the presence of *Candida a*.

We therefore wanted to verify how *Candida a*. can affect the levels of indoxyl sulfate, the result of our analysis is shown in the boxplot represented in Figure 11. In this case there is an opposite situation with respect to that shown in Figure 11 regarding the effects on 5-HT levels: The values of indoxyl sulfate expressed both in the 2nd and in the 3rd and 4th quartiles, are clearly higher in the case in which it is excluding the presence of *Candida a*..

We therefore wanted to investigate the relationship between *Candida a*. and cortisolemia, evaluating the ratios in the four peaks that are physiologically expressed in our organism at four distinct times of the day: 7, 13, 17, 23; here they are shown in the following boxplots in Figure 12. In all four comparisons it is evident that in the presence of *Candida a*. higher cortisol levels are on average associated with all four measurements. In this sense, it is interesting to remember how high levels of cortisolemia correspond to a condition of metabolic acidosis which is one of the conditions favoring fungal proliferation in the intestine. We then wanted to analyze the data obtained through the relationships between the values relating to the levels of EPA and DHA in the presence or absence of *Candida a*., The results are summarized in Figure 13, in this case the data show exactly opposite indications: While the presence of *Candida a*. it is accompanied by a decrease in the concentration of EPA, in the case of DHA the opposite trend is observed.

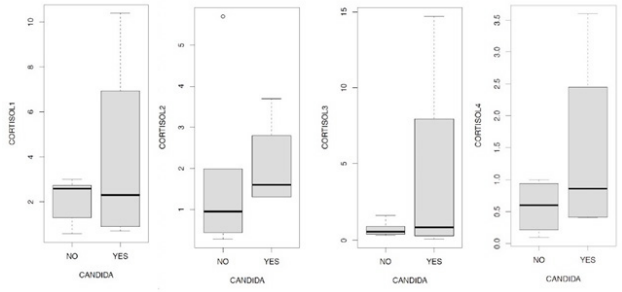

Figure 12. Relationship between *Candida a*. and cortisolemia. We wanted to investigate the ratios in the four peaks physiologically expressed in our organism at four distinct times of the day: 7.00 (cortisol 1), 13.00 (cortisol 2), 17.00 (cortisol 3), 23.00 (cortisol 4). In all four comparisons it is evident that in the presence of *Candida a*. higher cortisol levels are on average associated with all four measurements

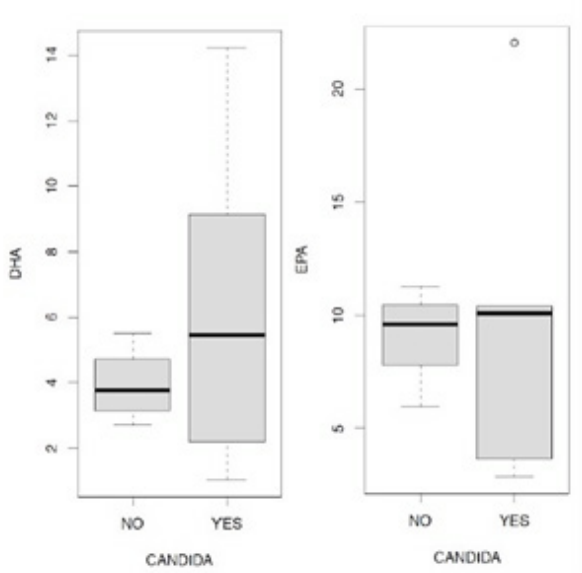

Figure 13. Relationships between the values relating to the levels of DHA (Figure 13.1) and EPA (Figure 13.2) in the presence or absence of *Candida a*.. The presence of *Candida a*. it is accompanied by a decrease in the concentration of EPA, in the case of DHA the opposite trend is observed

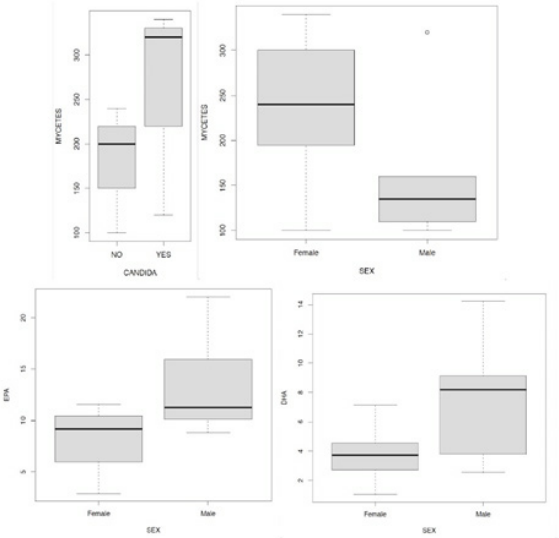

Figure 14. Relationship between the levels of *Candida a*. and mycetes. In all four quartiles in the presence of *Candida a*. higher levels of mycetes are associated with respect to subjects in which the fungus was absent. High

levels of fungi are mainly of fee relevance, as well as the lower levels of EPA and DHA seem prevalent typical of the women included in the study, as it shown in Figure 14.3/14.4

Another aspect to be carefully evaluated is that in the relationship that emerged between the levels of *Candida a*. and those of the mycetes measured in the subjects included in the study (Figure 14). From the boxplot it is clearly observed in all four quartiles as in the presence of *Candida a*. higher levels of mycetes are associated with respect to subjects in which the fungus was absent. It is interesting to note that from the analysis of the data obtained it seems that high levels of fungi are mainly of female relevance (Figure 14), as well as the lower levels of EPA and DHA seem, once again, prevalent typical of the women included in the study (Figure 14).

Subsequently, we wanted to describe the relationship between the levels of 5-HT with respect to the values of skatole, fungi, EPA and DHA (Figure 15) using as a discriminant of our analysis, the presence of low or high levels of the same 5-HT (with a new reference interval). From these data emerges strongly if on the one hand the skatole levels do not seem to influence those of 5-HT in any way (Figure 15), on the other we can observe how the low levels of 5-HT (Figure 15) are preferably associated with high values of mycetes (difference detectable in all quartiles represented in the two boxplots). Finally, low levels of EPA (Figure 15) are connected to low levels of 5-HT, a bond that we do not observe with DHA (Figure 15) where in the 2nd and 3rd quartiles low values of the second correspond to high levels of the 1st.

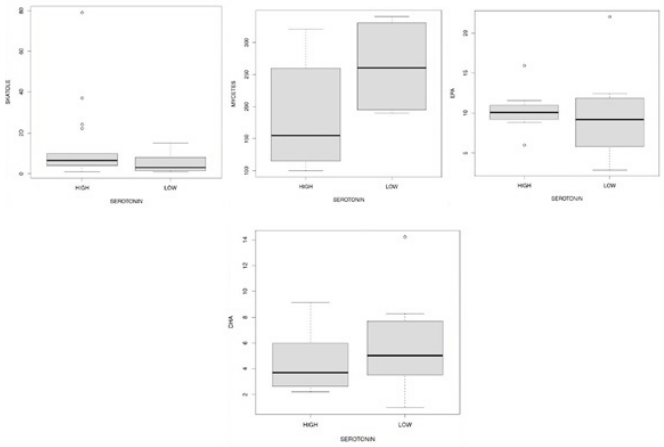

Figure 15. Relationship between the levels of serotonin with respect to the values of skatole, mycetes, EPA and DHA using as a discriminant the presence of low or high levels of the same serotonin

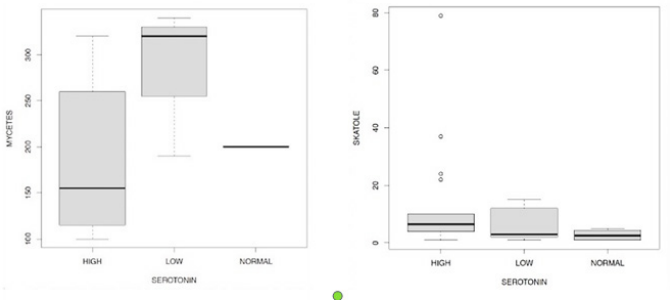

Figure 16. Relationship between the levels of serotonin with respect to the values of fungi and skatole using as a discriminant the presence of high, normal or low levels of the same serotonin. As it is possible to observe in Figure 16.1, a weak dependence emerges between the presence of skatole and the serotonin values, while it is interesting to remark how low serotonin levels are found in the presence of high levels of mycetes as it is shown in Figure 16.2

We wanted to deepen our evaluation of the results by dividing the 5-HT values into low, normal, high (following the old reference range) compared to the values of mycetes (Figure 16) skatole (Figure 16), cortisolemia-measured at 7, 13, 17, 23 (Figure 17), EPA and DHA (Figure 18). The evidences that emerged (Figure 16) showed the scarce dependence between the 5-HT values with respect to the influence of skatole highlight a dependence which in fact manifests itself weakly in case of low levels of the first compared to those of the second.

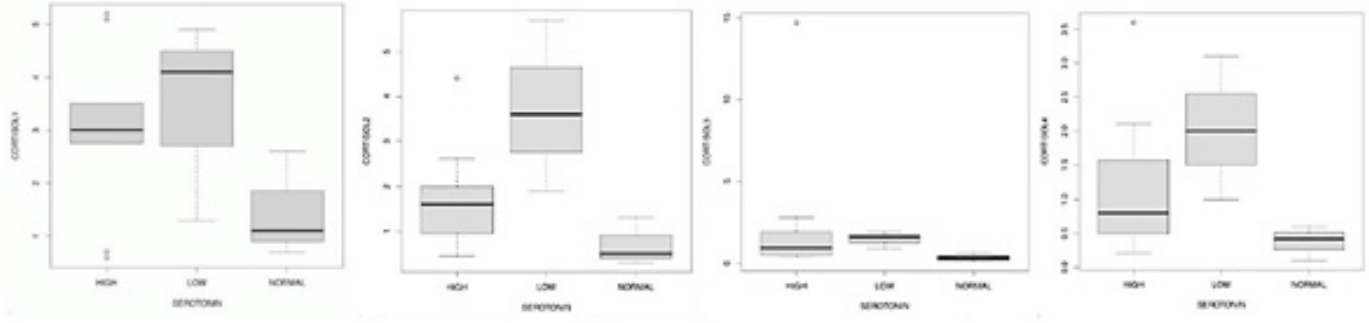

Figure 17. Correlations between the cortisol values, measured in the four different and physiologically significant intervals of the day (7.00, 13.00, 17.00, 23.00), and the serotonin values indicated in the reference intervals. we can easily observe an evident relationship between low serotonin levels and high concentrations of Cortisol-1/2/4

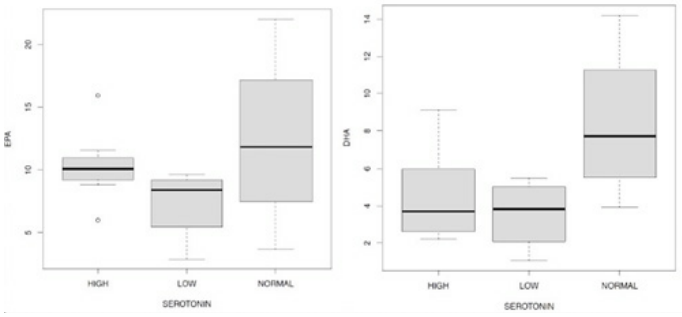

Figure 18. Relationship between the values of EPA and DHA compared to those of serotonin. We can observe how high levels of EPA and DHA are accompanied by normal concentrations of the neurotransmitter

It is very interesting, however, to observe the link between 5-HT and the levels of mycetes in Figure 16 which shows how low concentrations of the neurotransmitter are accompanied by high levels of the mycetes themselves. We wanted to verify any correlations between the cortisol values, still measured in the four different and physiologically significant intervals of the day, and the 5-HT values indicated in the reference intervals (Figure 17): We can see how there is an evident relationship between low 5-HT levels and high concentrations of Cortisol-1/2/4.

Finally we evaluated the relationship between the values of EPA and DHA compared to those of 5-HT and in this case we can observe an interesting fact: High levels of EPA and DHA are accompanied by normal concentrations of the neurotransmitter (Figure 18), in the case of the first fatty acid this is highlighted above all by observing the 2nd-3rd and 4th quartiles, while in the second this applies to all in and four quartiles represented in the boxplot.

In relation to what was previously illustrated and discussed about the sample examined, it is now desired to carry out a multivariate analysis that investigates, using a linear regression model, which are the main determinants that induce a variation in the levels of 5-HT in the subjects taken into consideration (Table 1). These variables are some of those discussed above. Their inclusion in the statistical model is, first of all, conditioned by the levels of correlation with 5-HT-the variables with the highest correlation or the mutable ones are selected that induce classes of distinct values according to the relative modalities-and, at the same time, to avoiding errors in interpreting the model, independent variables that are highly correlated with each other are not entered jointly. Following the construction of multiple models and robustness analyzes, it was decided to present the model shown in Table 1 formally expressed below:

Table 1. Multivariate analysis that investigates the main determinants that induce a variation in the levels of serotonin, the inclusion of the variables is conditioned by the levels of correlation with serotonin. From the study of these data we observe how men have a higher level of serotonin than women examined, cortisol 1 (measured at 7.00) and 3 (measured at 17.00) have a positive and statistically significant effect on serotonin, while the increase in cortisol 4 (measured at 23.00) there is a statistically significant reduction in serotonin values; furthermore in the presence of *Candida a*. and/or mycetes we registered a lower level of serotonin than in subjects who do not have *Candida a*. and/or mycetes. In the end, all three levels of indoxyl sulfate reported contribute to the determination of changes in serotonin in an inversely proportional way

Dependent variable	SEROTONIN
Sex (male)	1.292*** (0.125)
Cortisol 1	0.146*** (0.042)
Cortisol 2	-0.008 (0.040)
Cortisol 3	-0.090*** (0.016)
Cortisol 4	-0.237*** (0.074)
Indoxyl sulfate (medium)	-1.697*** (0.214)
Indoxyl sulfate (severe)	-0.963*** (0.215)
Indoxyl sulfate (slight)	-1.015*** (0.192)
Candida or mycetes (yes)	-0.320** (0.143)
Constant	4,565 (0.245)
Observations	12
Log Likelihood	-40.235
Akaike Inf. Crit.	100.470
Note: *p<0.1: "p<0.05: ***p<0.01	

The incidence of new thyroid function, adrenal hypofunction, and diabetes insipidus after surgery for Knosp1 and Knosp 2 pituitary adenomas is low. The pituitary-gonad axis has a lower postoperative remission rate than other hormone axes.

$$serotonin = \beta_0 + \beta_1 males + \beta_2 cortisol1 + \beta_3 cortisol2 + \beta_4 cortisol3 + \beta_5 cortisol4 + \beta_6 \text{indoxyl sulfate}\, severe + \beta_7 \text{indoxyl sulfate}\, medium + \beta_6 \text{indoxyl sulfate}\, minor$$

Where 5-HT represents the dependent variable while all the others are defined as independent variables. The βi coefficients represent the effect of the i-th independent variable in the determination of 5-HT. The greater the coefficient in absolute value, the stronger the link between 5-HT and the i-th independent variable. The sign of the coefficient identifies directly proportional (if positive) or inversely proportional (if negative) bonds.

Finally, remember that the categorical (or mutable statistical) variables-sex, *Candida a.*/mycetes, indoxyl sulfate-should be read marginally with respect to the value that does not appear, as it is captured by the intercept of the model (that is the constant β0). Therefore, from the study of what is reported in Table 1, the following emerges:

- Men have a higher level of 5-HT than women examined;
- In general, cortisol 1 and 3 have a positive and statistically significant effect on 5-HT, while the increase in cortisol 4 there is a statistically significant reduction in 5-HT values;
- In the presence of *Candida a*. and/or mycetes there is a lower level of 5-HT than in subjects who do not have *Candida a*. and/or mycetes;
- All three levels of indoxyl sulfate reported contribute to the determination of changes in 5-HT in an inversely proportional way, compared to subjects with eubiosis. These relations, statistically significant, have different magnitudes and in particular, the strongest effect is associated with the average indicate levels. Patients in our sample who have an average indoxyl sulfate level (according to reference to physiological levels) also have a much lower level of 5-HT. The empirical evidence goes in the same direction for the other two levels (mild and severe), however with a lower magnitude.

An impressive number of elements, referring to both experiments and clinical data, indicate that the microbiota plays a leading role within the GBA and that this role does not only imply its interaction with the ENS, but rather an exchange of information with the CNS through neuroendocrine and metabolic pathways. In particular in humans, the most stimulating indication of the existence of an intestinal interaction between the microbiota and the brain emerged about twenty years ago after the improvements found in patients with hepatic encephalopathy, after administration of antibiotics at the same time, the link between microbiota and anxiety-depressive syndromes emerged.

This study aimed to investigate the links between various parameters already associated with inflammatory diseases (such as the relationship between AA/EPA and AA/DHA) and others directly related to the stress axis (such as cortisol levels) with respect to intestinal dysbiosis (expressed through the indoxyl sulfate and skatole levels and the presence of *Candida a*. and/or mycetes) compared to the 5-HT levels considered in relation to CFS and depression.

From the data collected, and summarized in Table 2, we can make a series of observations. The research shows that of the 33 subjects included in the study, 88% have low levels of 5-HT which are related to mood and chronic fatigue; of these 33, 82% complained of morning fatigue, while in 79% of cases, indoxyl sulfate levels characteristic of a state of dysbiosis were measured, with 39% of subjects reporting swollen abdomen and in 52% alterations of the bowel habits. *Candida a*. and/or mycetes were isolated in the faeces of 67% of the sample under investigation. A side note regarding muscle contraction which was reported by 39%, a typical manifestation of metabolic acidosis.

Table 2. Main symptoms reported by the subjects included in the study. We examined 33 subjects with depression or CFS: 13 males (aged between 26 and 77 years) and 20 females (aged between 25 and 69 years)

Symptom	Number of patients	Percentage of the total number of patients
Abdonzinal swelling	13	39%
Tiredness in the morning	27	82%
Irregular alvo	17	52%
Neck/shoulders contracted	13	39%
Low serotonin	29	88%
High Indoxyl sulfate	27	82%
Presence of *Candida a*. or mycetes	22	67%
Cortisol 1	11	33%
Cortisol 2	12	36%
Cortisol 3	9	27%
Cortisol 4	11	33%

Conclusion

Although the results obtained are very interesting, and from many points of view encouraging specially for what emerged from the data analysis about the link between cortisol and 5-HT levels which highlight how there is a statistically significant link between these parameters, it is however necessary to collect a higher number of patients to obtain clinically meaningful indications. Even if the data shows the link between the levels of 5-HT and the values of indoxyl sulfate with respect to the presence of *Candida a*. or mycetes as well as the ratio between omega-3 and omega-6 we need to accumulate more data; in particular, the first three parameters seem to present a relationship as a function of the CFS or depression.

Statistics

The choice of tools for the statistical data analysis relative to the illustrated study, has been made difficult by different factors such as the lack of a control group and the limited number of cases studied; despite the difficulties, we tried to find a statistical significance in the data at our disposal using the following methodological approach.

The analysis of the collected data was conducted using graphical tools (boxplot also conditional on the different modalities of the mutable statistics available) and dependency measures (correlation between all the numerical variables collected). In particular, it was decided to build a correlation chart to have a quick view of the possible presence of a linear link between the observed variables.

The numerical variables for which physiological levels are also available were used for the construction of further boxplots in order to highlight any stylized facts otherwise not identifiable with the sole study of the correlations.

Furthermore, the preliminary descriptive analysis allows to highlight some distinctive features of the sample examined and to guide the consequent inferential analysis. Specifically, it was decided to conduct a more in-depth study based on the construction of a linear regression model in order to highlight, where possible, the main determinants of 5-HT. Particular attention was paid to the choice of variables in order to avoid violations of the assumptions of the model and, at the same time, problems of mispecification. In particular, this last aspect was taken care of avoiding the joint use of highly correlated variables in order to be able to interpret the marginal effect of each determinant on 5-HT well. It should be remembered that the analysis conducted was carried out only on patients without missing values in the variables considered. Furthermore, this is a preliminary study, whose empirical evidence, currently based on a limited number of observations, suggests interesting results to be explored in future research, enriching the sample of patients examined.

The estimates made using the linear regression model were subjected to statistical tests to evaluate their significance. Specifically, to test the null hypothesis under which it is assumed that the estimated parameter βi is equal to zero (absence of an effect of the dependent variable with respect to the dependent) against the alternative that is non-zero, a t-test was conducted. The latter shows that most of the independent variables are statistically significant with p-value <0.01.

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Conflict of Interest

The authors declared no conflict of interest.

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