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PROBIOSENIOR PROJECT:

ELDERLY PEOPLE AS A TARGET POPULATION FOR PROBIOTIC SUPPLEMENTATION AND FUNCTIONAL FOODS

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SUMMARY

In Europe the population of older people (65 years or more) is increasing significantly, because of the improved living conditions and life expectancy (EUROSTAT, 2019). At the same time, this demographic change leads to an increase of elderly people with age-related diseases. Therefore, the evaluation and monitoring over time of the well-being and health status of the population, especially of elderly, become of central importance for the modern societies. The main challenge is to study and develop new strategies and public actions to slow down the ageing process and increase the number of healthy seniors.

PROBIOSENIOR project comes from the need to answer this important issue, focusing the attention on the nutrition as important instrument to improve health status and general well-being. PROBIOSENIOR is a research project that aimed to evaluate the effects of a probiotic functional food-based diet on the reduction of low-grade inflammation in elderly subjects. It is a randomised, double-blind, placebo-controlled human trial that recruited 97 elderly healthy volunteers from boarding homes and private houses in Marche region (Italy), for the 3 or 6 months-administration of selected functional foods containing the probiotic mixture SYNBIO® (Synbiotec Srl, Camerino, Italy).

Lifestyle and eating habits information, psychological well-being and nutritional status data were collected through the administration of specific questionnaires, while biological parameters were monitored by collecting and analysing faecal, urine and blood samples. Two timepoints were considered - at baseline (T0) and after the dietary probiotic supplementation (T1) to monitor all variations during time.

Among the multidimensional tools to investigate a community, questionnaires are useful instruments to describe the population in all its aspects. The questionnaires selected for this project were: Lifestyle and eating habits questionnaire, Pathological anamnesis and Pharmacological therapy questionnaire, Mini Nutritional Assessment (MNA), The Psychological General Well-Being Index (PGWBI) and Bristol Stool Chart. The questionnaires revealed interesting outcomes. At T0, the MNA questionnaire showed most people at risk of malnutrition, while some improvements in nutritional status were observed in probiotic and placebo groups at T1. At the same time, an evident impairment was recorded in placebo-supplemented subjects respect to the others. The PGWBI

questionnaire described a population with moderate distress at T0; after the supplementation, a better improvement was observed in probiotic group (80% of cases) than in placebo one (50%).

Ageing is characterized by a low-grade chronic inflammation that involves several body systems and represents a risk factor for morbidity and mortality in elderly people. The biological parameters evaluated in this study are related to this condition, and they were: composition and diversity of gut microbiota, Short-Chain Fatty Acids (SCFA), biogenic amines (BAs), high sensitivity C-reactive protein (hsCRP), haematological parameters, cytokines and other inflammatory biomarkers.

Intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens; it can be shaped across the lifetime by modulation of the daily diet and supplementation.

In this study, the detected intestinal microbiota was typical for healthy elderly people, dominated largely by the phyla Firmicutes and Bacteroidetes, followed by Actinobacteria and Proteobacteria. At T1, statistically significant increase was observed for *Lactobacillus* spp. in probiotic group respect to placebo one, with a time-dependent improvement. Other significant changes were referred to *Staphylococcus* spp. and *Bifidobacterium* spp., which decreased respectively after probiotic and placebo supplementation. The 16S NGS analysis on probiotic group showed a decreasing trend of Proteobacteria at T1 and conversely, an increasing trend of Actinobacteria and Verrucomicrobia phyla, to which the increase of Akkermansiaceae and Bifidobacteriaceae contributes at the family level.

SCFAs are the main metabolites produced in the colon by bacterial fermentation. They are studied as potential mediators in the effects of gut microbiota on intestinal immune functions, but they are also involved in other physiological, behavioural and neurologic pathways.

In the present study, the subjects presented high variability between different genders and residence structures in SCFAs level at baseline. At T1 the main changes were referred to acetic and butyric acids, the most abundant SCFAs, with a greater increase in probiotic group than placebo one. The same trend was observed for total SCFAs, and considering the gradual reduction in the faecal concentrations of SCFAs with ageing, this is a promising outcome.

BAs are biologically active molecules, exerting essential physiological functions. Since BAs can be used as markers of different diseases, their detection and quantification are useful to evaluate the human health status.

In this study, the level of 11 BAs was determined in urine samples of the elderly volunteers. The results revealed a high inter and intra-variability at baseline and after the dietary intervention, with different effects exerted by the supplementations. Polyamines: spermidine levels positively decreased in probiotic group respect to placebo, at T1; putrescine and spermine decreased in both groups, despite the supplementation; on the contrary, cadaverine increased in probiotic group, decreasing in placebo group. Histamine and dopamine decreased after both supplementations; serotonin levels remained mainly unchanged in probiotic group respect to placebo; the opposite trend for tyramine amount that decreased. Phenylethylamine increased after both treatments. Finally, epinephrine and trymetilamine were not detected in the urine samples, because of their absence or their presence in quantities out of LOQ (limit of quantification) and LOD (limit of detection) ranges.

Ageing is also associated with a dysregulation of the immune system. Some cytokines are considered markers of inflammageing, age-related diseases and disability (muscle strength/power), but also longevity genes on ageing and life span. In our study, we determined the gene expression pattern of tumour necrosis factor alpha (TNF-α) and Insulin-like growth factor 1 (IGF-1) before and after the probiotics-based diet. Firstly, the results showed a significant increase in circulating levels of IGF-1 in probiotic group, respect to placebo-supplemented one. This outcome is in contrast with the expected reduction in elderly subjects, suggesting an IGF-1 modulation by GUT microbiota. Moreover, we evaluated TNF-α levels and its gene expression that resulted partially influenced by the dietary intervention. A significant increase was reported in mRNA expression of TNF-α in probiotic-supplemented group respect to placebo group. At the same time, the concentration of TNF-α decreased, although non significantly, in both groups, suggesting that the probiotic supplementation didn't directly affect this parameter.

Another marker of inflammation, but also an important risk factor associated with age-related diseases, is hsCRP. In this study, blood samples were collected to monitor hsCRP levels and general haematological parameters during the 24-week probiotic

supplementation. The main outcome was the significant reduction of hsCRP levels in probiotic group, respect to placebo; moreover, the 6-month-probiotic intervention resulted significantly more effective than the shorter one (<6 months). Blood parameters were not strongly affected by probiotic administration, although an improving in lipid profile was observed (P>0.05).

In conclusion, despite the limitations of the human trial and the complexity of biological pathways, PROBIOSENIOR project turned out to be an interesting strategy to maintain the host's well-being and prevent many age-related diseases in elderly. Overall, this study demonstrated that the probiotic-based intervention had effects on the modulation of gut microbiota and consequently of SCFAs' levels, suggesting its efficacy to prevent dysbiosis and modulate elderly health. Moreover, the supplementation significantly reduced hsCRP levels and modulated the GH/IGF-axis with potential positive effects on inflammation and pathogenesis of sarcopenia and other age-related morbidities. Also, the gene expression of TNF-α seems to be modulated, although without a direct effect on the reduction of pro-inflammatory cytokines. On the contrary, the probiotic supplementation influenced some BAs more than others, so further studies are needed to better understand the potential mechanisms by which probiotics modulate BAs. Finally, the results of questionnaires highlighted the benefits of this intervention on psychological well-being and nutritional status, improving the quality of life in the elderly community under study. Although they are preliminary findings that need to be confirmed, the interesting outcomes of PROBIOSENIOR project support, once again, the use of probiotics as a potential strategy to modulate elderly health.

In this project, we also developed the fundamental aspect of prevention and modulation of eating habits, through proper nutrition programs addressed to elderly people, caregivers and families. Practical education activities were developed, with the aim to raise people's awareness about nutrition and the use of probiotics, supporting and improving healthful behaviours, and preventing malnutrition. In this pilot program, we involved the community of two different boarding homes, and we used five videos on basic nutrition topics and final questionnaires to collect feedback about its impact and effectiveness on audience. This study revealed that this innovative communication method was a valid teaching approach for elderly. Moreover, most people recognized a positive impact of the program on their daily nutritional habits. In conclusion, this

intervention certainly contributed to improve the knowledge on nutrition and probiotics, in both elderly and caregivers, also providing a basis for designing effective nutritional education programs, specific for older adults.

CHAPTER I

GENERAL INTRODUCTION

1.1 THE AGEING AND LONGEVITY PHENOMENON

1.1.1 The global health: an overview

People worldwide are living longer, with a significant increase in the number of elderly people. There are different ways of defining older people, statistics on ageing generally categorize “older people” as being above a certain age threshold. Indeed, the United Nations (UN) noted in World Population Ageing 2019 that older people are commonly defined as those aged 60 or 65 years or more. World Health Organization (WHO) similarly states that older people in developed world economies are commonly defined as those aged 65 years or more, but it also shares an alternative definition, whereby an older person is defined as someone who has passed the median life expectancy at birth (EUROSTAT, 2020). This important increase in average life expectancy during the 20th century ranks as one of society’s greatest achievements. Today most people can expect to live into their sixties and beyond. Every country in the world is experiencing growth in both the size and the proportion of older persons in the population. By 2030, one in 6 people in the world will be aged 60 years or over. At this time the share of the population aged 60 years and over will increase from 1 billion in 2020 to 1.4 billion. By 2050, the world’s population of people aged 60 years and older will double (2.1 billion). Moreover, the number of persons aged 80 years or older is expected to triple between 2020 and 2050 to reach 426 million (Figure 1). Less developed regions of the world have experienced a steady increase in life expectancy since World War II, although not all regions have shared in these improvements. The most dramatic and rapid gains have occurred in East Asia, where life expectancy at birth increased from less than 45 years in 1950 to more than 80 years today. Although most babies born in 1900 did not live past age 50, in Japan, that is the current leader, life expectancy at birth now exceeds 83 years and is at least 81 years in several other countries (WHO, 2021).

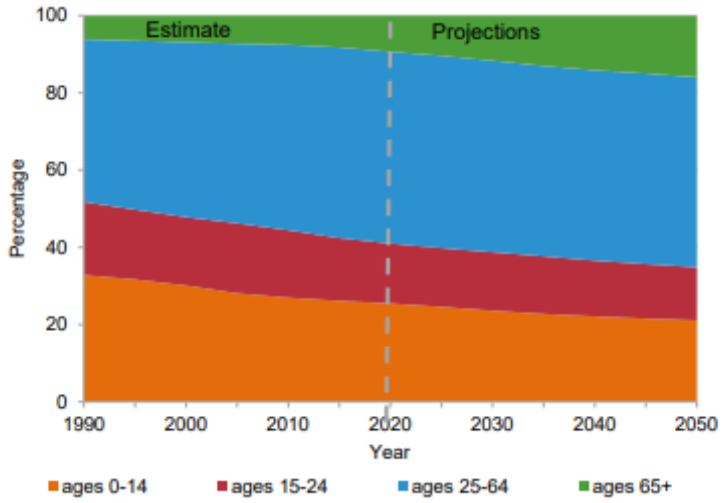

Figure 1. Global distribution of population by broad age group, 1990-2050 (United Nations Department of Economic and Social Affairs, population Division, *World Population Prospects 2019).*

This remarkable phenomenon is being driven by declines in fertility and improvements in longevity. With fewer children entering the population and people living longer, older people are making up an increasing share of the total population. In more developed countries, fertility fell below the replacement rate of two live births per woman by the 1970s, down from nearly three children per woman around 1950. Even more crucial for population ageing, fertility fell with surprising speed in many less developed countries from an average of six children in 1950 to an average of two or three children in 2005.

All these events are part of a major transition in human health spreading around the globe at different rates and along different pathways. This transition encompasses a broad set of changes that include a decline from high to low fertility, a steady increase in life expectancy at birth and at older ages and a shift in the leading causes of death and illness from infections, parasitic diseases and chronic conditions. In early non-industrial societies, the risk of death was high at every age and only a small proportion of people reached old age. In modern societies, most people live past middle age, and deaths are highly concentrated at older ages. The victories against infections and parasitic diseases are a triumph for public health projects of the 20th century, which immunized millions of people.

Another important point has been the improvement of life quality and resources that allowed to reduce serious infections and prevent deaths among children. Better living standards, more nutrient diets, advances in public health and medical technologies, increased awareness of the benefits linked to a healthy lifestyle, a move away from heavy labour towards tertiary occupations and improved living conditions further contributed to the improvement of life expectations for all people. The progressive increase in survival in these oldest age groups was not anticipated by demographers, and it raises questions about how high the average life expectancy can realistically rise and about the potential length of the human lifespan (EUROSTAT, 2020).

While population ageing is a global phenomenon, the ageing process is more advanced in some regions of the world than in others. The pace of population ageing in many developing countries is substantially faster than the historical precedents observed in developed economies. In many countries, the 85-and-over population is projected to increase 351 percent between 2010 and 2050, compared to a 188 percent increase for the population aged 65 or older and a 22 percent increase for the population under age 65. The global number of centenarians is projected to increase 10-fold between 2010 and 2050 (EUROSTAT, 2019).

Behind Japan, the EU provides one of the most distinctive examples of demographic ageing. The G20 countries are at various stages of economic and population development. While the process of population ageing is particularly established in regions like Japan, Northern Africa, Asia, Latin America and the Caribbean (U.N., 2019), this may be contrasted with South Africa or Saudi Arabia where young people dominate the population profile. In 2015, older people accounted for 8.2% of the world's population. At one end of the spectrum, the share of older people was more than three times the global average in Japan, where the share of people aged 65 years or more in the total population was more than one quarter (28.0% in 2018). The EU-27 had the next highest share of older people among the G20 nations (20.3% in 2019). Half of the remaining non-EU G20 countries had shares of older people in their total populations that were above the global average; these included United States (16.0% in 2018) and China (9.3% in 2015). The G20 countries where older people accounted for a relatively small proportion of the total population are often characterised as emerging economies, with relatively young populations and expanding labour forces; examples include Mexico (where older people

accounted for 7.4% of the total population in 2019), India (5.6% in 2015), Indonesia (5.4% in 2015), South Africa (5.0% in 2015) and Saudi Arabia (3.0% in 2015). In 2020, the median age of the world population is projected to be 30.9 years. Japan (48.4 years) had the highest projected median age among the G20 nations and was followed by the EU-27 (43.9 years) (Figure 2). There were only four G20 countries where the median age was below the world average: Indonesia, Mexico, India and South Africa (United Nations, 2019a, b).

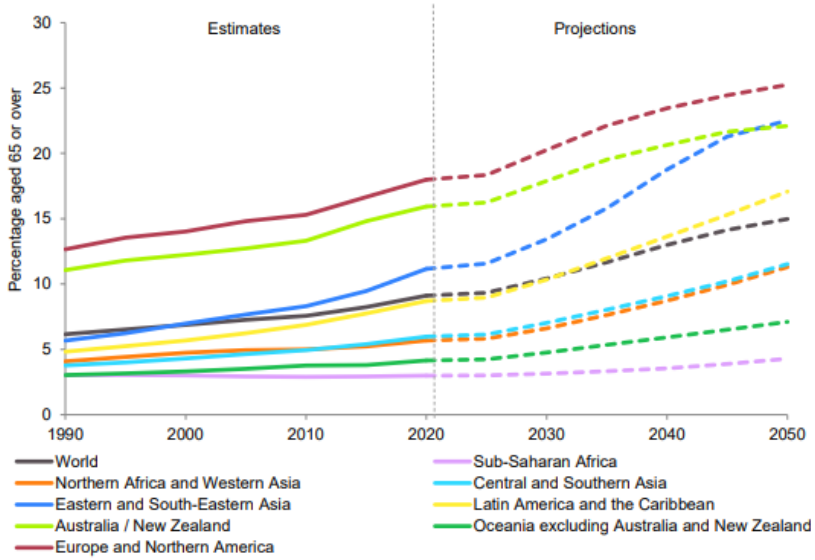

Figure 2. Share of total population aged 65 years or over, by region, 1990-2050 (United Nations Department of Economic and Social Affairs, population Division, *World Population Prospects 2019).*

Epidemiological studies also showed a set of projections about the median age: by 2050, the median age of the world population is projected to reach 36.2 years. The highest median ages are projected for eastern Asia, peaking at 56.5 years in South Korea and 54.7 years in Japan, while the median age of the population in China (47.6 years) is projected to rise to almost the same level as in the EU-27 (48.2 years). By 2050, the only G20 country where the median age is projected to remain below the world average is South Africa (33.9 years) (United Nations, 2019b).

In eastern Asia, the process of population ageing is rapid. The old-age dependency ratio for Japan is projected to continue rising at a rapid pace through to 2050, when it is

projected to reach 80.7%. This implies that, having had almost four working-age people (defined here as those aged 20-64 years) for each older person (aged 65 years or more) in 2000, Japan will move to a situation of having approximately 1.2 working-age persons for every older person by 2050. During the next three decades there will also be a considerable shift in the structure of the Chinese population. From having an old-age dependency ratio that was close to but just below the world average in 2015, China is projected to see its ratio rise rapidly such that it will be 1.7 times the level projected for the world by 2050 (WHO, 2021).

1.1.2 The European situation

Population ageing is a long-term development that has been apparent for several decades in Europe. This process is being driven, as in most of the world countries, by historically low fertility rates, increasing life expectancy and, in some cases, migratory patterns.

There were 90.5 million older people (aged 65 years or more) living in the EU-27 at the start of 2019; this equated to approximately one fifth (20.3%) of the total population.

Population projections suggest that the ageing of the EU's population will quicken in the coming decades, with a rapid expansion in the number and share of older people (EUROSTAT, 2020).

The total population of the EU-27 is projected to increase marginally from 446.8 million at the start of 2019 to peak at 449.3 million during the period 2026-2029, before falling slowly back to 441.9 million by 2050. Consequently, the population of older people (aged 65 years or more) in the EU-27 will increase significantly, rising from 90.5 million at the start of 2019 to reach 129.8 million by 2050. During this period, the number of people in the EU-27 aged 75-84 years is projected to expand by 56.1 %, while the number aged 65-74 years is projected to increase by 16.6 %. By contrast, the latest projections suggest that there will be 13.5% fewer people aged less than 55 years living in the EU-27 by 2050.

The age profile of society is rapidly developing, so that the relative importance of the very old (people aged 85 years or more) is growing at a faster pace than any other age group and by 2050 there will be close to half a million centenarians.

The median age of a population provides a useful summary of the overall age profile, and it is going to increase rapidly until 2050. A range of factors may influence the median

age, including fertility, life expectancy, social and economic development. In 2019, the median age of the EU-27 population was 43.7 years. Across the EU Member States, the median age was below 40.0 years in Luxembourg (39.5 years), Cyprus and Ireland (where the lowest median ages were recorded, both 37.7 years). By contrast, the median age of the population was considerably higher in Germany (46.0 years) and peaked in Italy (46.7 years). The EU-27's median age is projected to increase by 4.5 years during the next three decades, to reach 48.2 years by 2050. An increase is projected in each of the EU Member States, with the median age of the population projected to rise by more than 8.0 years in Poland, Slovakia and Malta. At the other end of the range, the age profiles of France, Belgium, the Netherlands and Denmark are projected to develop at a slower pace, as their median ages are projected to increase by 3.0-4.0 years during the period under consideration. The pace of change is projected to be even slower in Sweden (where the median age is projected to increase by 2.6 years) and particularly Germany (a projected increase of 1.2 years).

In 2019, the share of the very old people in the EU-27 population was 2.8%. There were five EU Member States where this share was less than 2.0%, with Ireland, Cyprus and Slovakia registering the lowest shares (1.5-1.6%). By contrast, France and four southern Member States (Portugal, Spain, Greece and Italy) had the highest shares of very old people, with a peak of 3.6% recorded in Italy. There were more very old women than very old men in each of the EU Member States: however, the share of very old men was generally rising at a faster pace than the share of very old women between 2001 and 2019.

As mentioned before, distribution of elderly people between the two sexes is not homogeneous, women outnumber men at older ages within the EU-27 population. In recent years, this gap has started to narrow, as an increasing number of men survive to older ages. In 2019, there were, on average, 1.33 women aged 65 years or more in the EU-27 for every man of the same age. The biggest gender imbalances were recorded in the Baltic Member States (EUROSTAT, 2020).

1.1.3 The Italian situation

According to the Istat, the National Institute of Statistics, Italy together with France, has the record number of more than 100 years of age among European countries. Italy

can indeed boast of one of the world's most advanced health care systems, lifestyle and a healthy dietary model based on the Mediterranean diet, making it one of the countries where life expectancy is among the highest (Istat, 2019).

Therefore, Italy has the third oldest population in the world. As of 2020, 23 percent of the Italian population were aged 65 years and older, the same as Martinique, after Japan and Monaco. As shown in the figure 3, the average age of the Italian population is 45.7 years and has constantly been increasing in recent years, with this projected to grow further in the coming decades. The oldest regions of the country are Liguria and Friuli-Venezia Giulia, both situated in the North, while the youngest regions are Campania, in the South, and Trentino-South Tyrol, in the North-East (Statista Research Department, 2021).

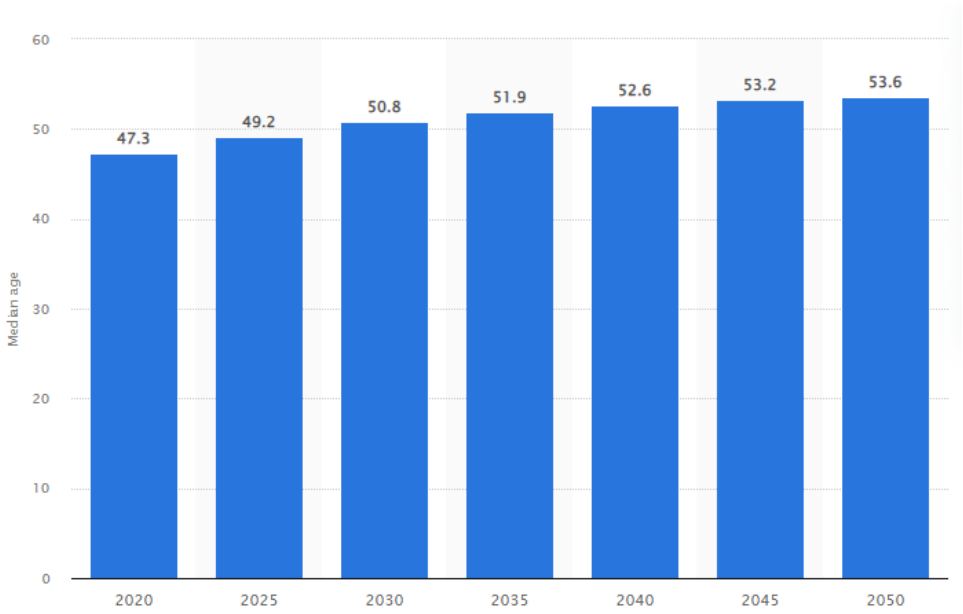

Figure 3. The average age of the Italian population, 2020-2050 (STATISTA, 2021).

According to the previous Istat study of 2017, the expected resident population in Italy would be 58.6 million in 2045 and 53.7 million in 2065; moreover, in 2065 the average life would increase to 86.1 years for men and up to 90.2 years for women, compared to an average life of 80.1 and 84.6 years respectively in 2015 (Istat, 2017).

As for other countries, the main explanations for Italy's ageing population are a high life expectancy and a low birth rate. The result is that people live longer, alongside a declining number of births. Indeed, Italy is among the countries with the highest. For

females, it reached 85 years, while for males 81 years. In addition to this, it ranks among the countries with the lowest fertility rates in the world. However, there are some regional differences. The birth rate is highest in the Southern regions and on the Islands, while it is lowest in the Central and North-Western regions. The median age of the population in Italy is estimated to grow constantly until 2050. Projections made in 2019 by Statista Research Department suggested that the median age will be equal to 50.8 years in 2030 and 53.6 years by 2050. In Italy, the birth rate has constantly decreased over the past years. In 2019, roughly seven children were born per every 1,000 inhabitants, about two infants less than in 2002. On the other hand, the number of deaths in Italy remained rather stable since the beginning of the twenty-first century. This environment changed in 2020, when the coronavirus (COVID-19) started to spread among the Italian population. The death rate reached 12.6 per 1,000 inhabitants, a notable increase compared to previous years. From the perspective of the single regions, the highest number of deaths was registered in Liguria, whereas the lowest death rate in the country was reported in Campania (Statista Research Department, 2021).

1.1.4 Marche region trend

The study “Argento-Marche”, conducted in 2003, found that in Marche region people aged 65 and over represent 21% of the population: they were almost 316,000, of which 133,000 men and 183,000 women.

According to data updated to 2020, Marche region is in eighth position in the ranking of Italian regions with the highest number of elderly people. In Marche region there were 202.3% elderly people every 100 young individuals. They are progressively increasing and it has expected that in 2023 would be more than 400,000. Of those interviewed, 36% considered their health good, 48% considered their health discreet and 15% considered their health bad; older people who had a negative perception of their health status were those over 74 and women felt less healthy than men.

From the “Argento study”, the state of health of the elderly population in Marche Region was overall good, however data confirmed that almost half of the sample had three or more chronic pathologies. Moreover, 3% of the sample was not self-sufficient and another 20% of respondents needed help in daily life activities; in addition, 26% of

the tested population were positive for screening for cognitive disorders (Mancini et al., 2003).

1.2 INFLAMMAGEING

1.2.1 Inflammageing

Ageing is a ubiquitous complex phenomenon that results from environmental, genetic, epigenetic and stochastic events in different cell types and tissues and their interactions throughout life. A pervasive feature of ageing tissues and most age-related diseases is chronic inflammation (Franceschi and Campisi, 2014).

To better describe this condition, Franceschi *et al.* in 2000 coined the term "Inflammageing", representing a newer dimension to ageing studies and decrees that the ageing process has a chronic progressive proinflammatory phenotype (Kirkwood, 2018).

There are several theories and terminology to explain inflammageing phenomenon, although sometimes there is a disagreement in the definitions. Briefly, from a theoretical perspective, inflammageing is the expansion of the network theory of ageing and the remodelling theory of ageing (Franceschi, 1989; 1995). The network theory of ageing hypothesizes that ageing is indirectly controlled by the network of cellular and molecular defence mechanisms. The remodelling theory is the gradually adaptive process of the body with a net result controlling malignant damage resulting in a trade-off with immunity; therefore, when innate immunity is activated causing an increased pro-inflammatory state can be termed inflammageing. The remodelling theory was also implied to explain immunosenescence (Salminen et al., 2008). Others proposed the oxidation-inflammation theory of ageing (De la Fuente and Miquel, 2009).

The prevailing consensus is that the primary feature of inflammageing is an increase in the proinflammatory status with advancing age (Kirkwood, 2018). More completely, inflammageing describes the low-grade, chronic, systemic inflammation that develops with advanced age, in the absence of overt infection ("sterile" inflammation). Inflammageing plays an increasingly important role in the rate of ageing and age-related diseases, and it is a highly significant risk factor for both morbidity and mortality in elderly people. In some cases, it may contribute to clinical manifestations of other age-related pathologies (Franceschi and Campisi, 2014).

This topic is attracting a lot of interest, due to its impacts on society and on the quality of life of the population, enough to increase the research activities to discover and investigate all aspects of this phenomenon, to better understand causes, effects and potential solutions and any preventive strategies.

1.2.2 Inflammageing causes

The aetiology of inflammageing and its potential causal role in contributing to adverse health outcomes remains largely unknown. The identification of pathways that control age-related inflammation across multiple systems is therefore important in order to understand whether treatments that modulate inflammageing may be beneficial in people (Franceschi and Campisi, 2014). There are several potential mechanisms and sources of inflammageing, they are not mutually exclusive, and their relative contributions require further studies.

One source of inflammageing could be the damaged macromolecules and cells (self-debris) that accumulate with age due to increased production and/or inadequate elimination. Self-debris released as a consequence of cell/organelle injury can mimic bacterial products and function as endogenous “damage”-associated molecular patterns that activate innate immunity. Molecules and substances like damaged cellular and organelle components, free radicals from oxidative stress, metabolites (extracellular ATP, fatty acids, urate crystals, ceramides, cardiolipin, amyloid, succinate, peroxidised lipids, advanced glycation end-products) are recognized by a network of sensors as “danger” signals and initiate immune reactions that are necessary for physiological repair. However, as damage accumulates, the danger responses can become chronic and hence maladaptive.

A second source of inflammageing might be represented by metabolites and other products released by oral and gut microbial population, which can leak into surrounding tissues and the circulation. The permeability of the intestinal barrier decreases with ageing and consequently the probability of passage of active compounds and metabolites increases leading to chronic low-grade inflammation. Alternatively, the gut microbiota itself might change with age so that the microorganisms present in the aged gut elicit an inflammatory response. This phenomenon doesn’t occur in young gut, for this reason

host–pathogen balance is important at every age to keep “harmful” agents inactive (Franceschi et al., 2000; 2014).

Third, inflammageing might be due to cellular senescence. Senescence is a cellular response to damage and stress conditions; it suppresses the proliferation of cells with a compromised genome and contributes to optimal wound healing in normal tissues. Furthermore, persistent senescent cells are thought to guide ageing and age-associated diseases through their secretory phenotype and they tend to accumulate with age in many tissues. Practically, senescent cells feed age-related pathologies because they secrete numerous proinflammatory cytokines (senescence-associated secretory phenotype or SASP) that modify the tissue microenvironment and alter the function of nearby normal or transformed cells. Studies revealed that the removal of senescent cells in prematurely aged mice prevents several age-related pathologies (Coppé et al., 2010). Senescent cells tend to accumulate in adipose tissue, particularly the visceral fat of obese individuals; fat is a rich source of inflammatory cytokines and major changes in fat distribution and lipid composition may have important clinical consequences linked to age-related disorders (Tchkonia et al., 2010).

Inflammageing could be also caused by the increasing activation of the coagulation with age since coagulation and inflammation share components and strong interactions. An example is the increased risk of hypercoagulation observed with ageing that may account for the higher incidence of arterial and venous thrombosis in the elderly people (Franceschi and Campisi, 2014).

Another important phenomenon is that age-related changes to the immune system (immunosenescence) could contribute to inflammageing, since adaptive immunity declines with age, whereas innate immunity undergoes more subtle changes that could result in mild hyperactivity. These age-related changes most likely result from both lifelong exposure to pathogens and antigens, as well as intrinsic changes in immune cells and possibly genetic predisposition. A major role is likely played by persistent infections such as those caused by CMV and HIV, which are associated with accelerated immunosenescence and ageing. Finally, defective, or inappropriate regulation of the complement pathway can lead to local inflammatory reactions in age-related macular degeneration, the leading cause of blindness in the elderly people (Gallenga et al., 2014).

This mechanism could be involved in many other degenerative diseases (Franceschi and Campisi, 2014).

1.2.3 Inflammageing biomarkers: C-reactive Protein

Biomarkers can be defined as any alterations in the constituents of body or tissue fluids. These kinds of markers provide a useful instrument for uniform classification of a disease with its risk factors and can be extended in understanding the basic underlying physiopathology of disease. Furthermore, a biomarker is a parameter (chemical, physical, or biological) that can be applied not only to detect and monitor the progress of disease but also the effectiveness of treatment in preclinical research and clinical diagnosis (Ansar and Ghosh, 2016). More significantly, a biomarker points out a change in state or expression of proteins, peptides, genes, and other factors that associate with the progression or risk of a disease, initial diagnosis, drug response, susceptibility of the patient to a given treatment, drug target identification, or disease intervention. Advances in research tools allow to study the whole set of biomarkers using proteomics technologies, genomics, different imaging technologies, and invasive or non-invasive laboratory investigations (Kumar and Sarin, 2009; Mayeux, 2004).

Humans and animals are continuously exposed to trauma, stress and injury that can stimulate the organism to elicit a set of highly organized immunological, physiological, metabolic, and behavioural responses. Since the causes and sources of inflammation are manifold, also the involved groups of mediators, biomarkers, pathways, and key responders/effectors during the immune response inflammation are varied. In laboratories, animal models for inflammatory diseases have been developed to understand how inflammation process works, identify inflammatory biomarkers and find out their potential role in therapeutics.

The biomarkers responsible for inflammatory diseases are of heterogeneous nature, but currently, there are no specific markers for inflammation. In hospitals some broad-spectrum inflammatory markers are routinely investigated and could be identified for safe assessment in diagnosis, treatment and prognosis which may be helpful for routine diagnosis in the future (Ansar and Ghosh, 2016). Concerning inflammation, the clinical investigation revealed some broad-spectrum markers like C-Reactive Protein (CRP), erythrocyte sedimentation rate (ESR), total leukocyte count (TLC), differential leukocyte

count (DLC), sodium–potassium level, urea–creatinine level, antinuclear antibody, pro-brain natriuretic peptide (pro-BNP), and human leukocyte antigen (HLA) (Ansar and Ghosh, 2016). Perhaps the most regularly used nonspecific prognostic inflammatory biomarker is the C-Reactive Protein. CRP, named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, was the first acute-phase protein to be described and now it is an important sensitive systemic marker in the diagnostic of a varied spectrum of diseases. Human CRP is composed of five identical, non-glycosylated polypeptide subunits, each composed of 206 amino acid residues, but a CRP monomeric structure has been also described and this form seems to have greater prothrombotic properties (Molins et al., 2008;2011). It is a general marker in inflammation that reveals when a pathological condition is related to an inflammatory or non-inflammatory state. The CRP level can also define if the inflammation is acute (severe and sudden, such as with an allergic reaction) or chronic (persistent, such as in elderly people). The assessment of disease pathology, diagnostics, prognostics, and therapeutic applications of CRP in different diseases is already cited (Ansar et al., 2006; 2013).

Recent studies have also provided evidence of systemically generated inflammatory biomarkers versus mediators both in acute and chronic inflammation, although these kinds of markers are often examined one by one. The process of inflammation is triggered by mononuclear phagocytes, which in turn alter the concentration of various biomarkers, the cascade of complement pathways, antibody production, and subsequent tissue repairing and resolution, when possible. This complex process, the relationship between biomarkers or with mediators remains still largely unclear, while it is an important aspect to study and deepen (Ansar and Ghosh, 2016).

As described in the previous paragraphs, inflammageing is the condition developed by most older people and it is associated with dysregulation of the inflammatory response by immune system (Baylis et al., 2013). In the phenomenon of inflammageing, the remodelling of the innate and acquired immune system plays an important role, resulting in chronic inflammatory cytokine production. Inflammatory biomarkers produced during this long-lasting flogistic process could also carry high susceptibility to chronic morbidity, disability, frailty, and premature death; since it is a trigger for further adverse events, chronic inflammation process is an event not to be underestimated in the elderly population (Molins et al., 2008).

Ageing is defined by an evident inflammatory state with high circulating levels of pro-inflammatory markers. In addition to the CRP, there is a list of markers involved in inflammageing, such as IL-1, IL-1 receptor antagonist protein (IL-1RN), IL-6, IL-8, IL-13, IL-18, IFNα and IFNβ, transforming growth factor-β (TGFβ), tumour necrosis factor (TNF) and its soluble receptors (TNF receptor superfamily members 1A and 1B) and serum amyloid A. Although it's still difficult to make a comprehensive list of pro-inflammatory markers tightly associated with ageing, high levels of the markers just cited are detected in the majority of older individuals, even in the absence of clinically active diseases (Ferrucci and Fabbri, 2018).

The CRP is the main inflammatory marker even in older subjects, where, in case of inflammatory process ongoing, stimulus by IL-6 and other cytokines causes the release of the CRP by hepatocytes. Several studies have shown that this protein, in form of High-Sensitivity C reactive protein (hsCRP) can be considered a valid and reliable marker for the diagnosis of several pathologies, at the base of which there is the establishment of an inflammatory process and/or tissue damage. This feature does not belong to all other markers. Increased CRP production, for example, seems to be associated with acute myocardial infarction and acute coronary syndrome, since tissue necrosis is a potent acute-phase stimulus and, following myocardial infarction, there is a major CRP response which level reflects the degree of myocardial necrosis (Pepys and Hirschfield, 2003). Some studies revealed that increased levels of CRP are also associated with depression, there is a relationship between hair cortisol and plasma CRP with the persistence of depressive symptoms, across a 14-year period in older adult population; so elevated cortisol and CRP levels could be reliable biomarkers of somatic depressive symptoms (Iob et al., 2020). Moreover, there is growing evidence that hypertension, atherosclerosis, and vascular disease in general are inflammatory diseases and therefore associated with CRP increment (Hage, 2014). Other pathological conditions related with high levels of CRP are age-related macular degeneration (AMD), that is a condition characterized by central vision loss in elderly individuals and osteoarthritis, a chronic joint disorder with low grade systemic inflammation (Chirco et al., 2016; Jin et al., 2015). Finally, Chambers *et al.* (2001) found further strong positive correlations between baseline CRP concentration and BMI: the weight loss lowers the CRP value. Raised CRP values are also associated with the insulin resistance or metabolic syndrome. Physical exercise and moderate alcohol

consumption are both associated with the lowering of base-line CRP concentration; in contrast, there is a positive association of CRP values and smoking (Pepys and Hirschfield, 2003).

1.2.4 Chronic low-grade inflammation in elderly people and geriatric syndromes

The ageing phenotype can be described as a complex mosaic resulting from the interaction of a variety of environmental, stochastic, genetic and epigenetic events/stimuli. In its broadest sense, ageing refers to the changes that occur during an organisms' life span, involving combinations of molecular, cellular and physiological variables, and a small number of basic molecular mechanisms, including some evolutionary highly conserved basic biological mechanisms responsible for body maintenance and repair (Colloca et al., 2020). The changes that continuously occur in human body could be innocuous or, in case of ageing, lead to increased risk of disease, disability, or death; in this case the proper term to use is "senescence". Senescence is defined as the progressive deterioration of bodily functions over time and normal human ageing has been correlated with a loss of complexity in a wide range of anatomic structures and physiological processes, such as blood pressure, respiratory cycles, vision, postural dynamics and increased risk of mortality. There is no clear evidence which changes are the most important drivers of the ageing process and how they influence each other, but one of the key mechanisms is inflammation (Colloca et al., 2020). Actually, inflammation could be beneficial and useful when it is an acute and transient immune response to harmful conditions such as traumatic tissue injury or an invading pathogen (Franceschi and Campisi, 2014). This response also facilitates the repair, turnover, and adaptation of many tissues. However, during ageing, this kind of responses may be impaired, leading to the development of inflammageing.

This phenomenon results as critical trigger in the pathogenesis of major age-related chronic diseases, such as changes in body composition, energy production and utilization, metabolic homeostasis, osteoporosis, type 2 diabetes, atherosclerosis, neurodegeneration, disability, cardiovascular diseases thus contributing to premature mortality (Franceschi and Campisi, 2014). Furthermore, in elderly people, tissues (adipose tissue, muscle), organs (brain, liver), systems (immune system), and ecosystems (gut microbiota) of the body can contribute to the onset and progression of the systemic

inflammatory state. Epidemiological studies have proved that inflammageing plays a crucial role in the most important geriatric conditions that could seriously interfere with a person's daily life (Colloca et al., 2020); based on these findings, many researchers have proposed that inflammageing could be used as marker of accelerated ageing and should be considered to be one of the pillars of the biology of ageing (Ferrucci and Fabbri, 2018).

The world of geriatric syndromes is wide and continuously object of study; by definition, a "geriatric syndrome" is a clinical condition typical of elderly individuals characterized by multiple causes determining a unified manifestation. It includes a group of signs and symptoms occurring together and characterizing an abnormality. This implies that geriatric syndromes present a multifactorial and extremely heterogeneous background, causing clinical, psychological, social, and environmental vulnerabilities (Inouye et al., 2007). One of the most problematic expressions of population ageing is frailty. It is a geriatric syndrome characterized by the reduction of functional reserves and increased vulnerability to stressors, resulting from the cumulative decline of multiple physiological systems that cause an increased risk of adverse health-related outcome (Cesari et al., 2017). Frailty is sometimes presented as a syndrome, some others as a state of health determined by the age-related accumulation of deficits; this ambiguity depends on the patient's overall state when it is diagnosed. Generally, frailty is frequently described as a syndrome because associated with the widely diffused model proposed by Fried and colleagues (2001) in which, the so-called frailty phenotype, is based on the assessment of 5 criteria: weakness, slowness, exhaustion, low activity and weight loss (Fried et al., 2001). More recently, in France, has been developed a frailty screening tool based on the frailty phenotype that includes also social and cognitive factors, modifying the definition of this geriatric situation as "a medical syndrome with multiple causes and contributors that is characterized by diminished strength, endurance and reduced physiologic function that increase an individual's vulnerability for developing increased dependency and/or death" (Morley et al., 2013). Among the geriatric events, it is important to describe the most common conditions. *Falls:* defined as an event which results in a person coming to rest inadvertently on the floor, causing distress for the individual due to the consequent higher morbidity, loss of physical function, and increased risk of death. In advanced age, falls have multifactorial aetiology, including the age, sensory impairment, musculoskeletal weakness, postural modifications and

hypotension, medications, and environment. *Cognitive impairment*: defined as deterioration in memory or executive function, difficulty learning new information or recalling previously learned information and other disturbances of cognitive function such as aphasia, apraxia, and agnosia (Kane et al., 2012). Dementia is the most common cognitive impairment; some symptom clusters are called dementia subtypes, such as Alzheimer's disease, frontotemporal dementia (FTD), vascular dementia and Lewy body dementia (Finucane, 2018). *Disability:* defined as having difficulty with or requiring help with ADLs (dressing, hygiene, bathing, toileting, transferring, ambulating, feeding, and grooming) or IADLs (telephoning, shopping, preparing meals, housekeeping, transportation, medication, and financial management) (Kane et al., 2012). The age-related impairment of movement is multi-causal even if the quantitative and qualitative decline of skeletal muscle (sarcopenia) may represent one of the main causes of the loss of mobility. Finally, *Malnutrition*: defined as undesirable weight loss or low body mass index (BMI). Among the contributing factors, dysphagia and oral health problems such as tooth loss, toothache and difficulty chewing play a key role in malnutrition in elderly people Khan et al., 2014; Van Lancker et al., 2012). Malnourished older people are characterized by some biochemical markers, such as low blood albumin levels, anaemia, and deficit of micronutrients (iron, B12 or folate deficiency) that should monitored (Kane et al., 2011).

1.3 NEW STRATEGIES FOR HEALTHY AGEING

1.3.1 The new concept of healthy ageing

The substantial increases in life expectancy at birth achieved over the previous century, the medical advances and the increase in health care costs, have led to international interest in how to promote a healthier old age and how to age “successfully” (Bowling and Dieppe, 2005).

The term “successful ageing” refers to an idea of ageing characterised by life satisfaction, longevity, freedom from disability, mastery and growth, active engagement with life and independence (Martin et al., 2015). Behind this, there is also the philosophy of “active ageing” that underlies, therefore, that ageing should not be considered as the final stage of life but as a period during which older people contribute to society and

should therefore be preserved. Active ageing is an important concept that can bring benefits for the individual and the community, but also for the whole society since elderly population in healthy state, that is autonomous and integrated, is a population less in need of health care (WHO, 2012).

Active ageing seems to refer to a positive state of elderly people with absence or reduced presence of disease, but this concept needs to be expanded. Most health problems are the result not only of the presence of chronic diseases coexisting in the same subject, but also of their interaction with the ageing process itself, resulting in modification of the functional capacity that could influence life's habits. It is for this reason that the World Health Organization in its World Report on Ageing and Health, defined and extended the concept of Healthy Ageing to the process of developing, promoting, and maintaining the functional ability that allows well-being in old age. With this new concept, ageing in a healthy way does not mean ageing without disease, but being able to do things that we value for as long as possible. This includes some personal abilities like meet their basic needs; learn, grow and make decisions; be mobile; build and maintain relationships; contribute to society. Functional ability consists of the intrinsic capacity of the individual and it is influenced by environmental characteristics and the interaction between them. Intrinsic capacity results in all the mental and physical abilities that a person can draw on and includes their capacity to walk, think, watch, hear and remember. The level of intrinsic capacity is influenced by several factors such as the presence of diseases, injuries and age-related changes. While environments include the home, community and broader society; also, all the factors within them such as the built environment, people and their relationships, attitudes and values, health and social policies take part to this aspect (WHO).

1.3.2 New strategies for healthy ageing

The ageing and the longevity of population have become the global public health challenge because, for the first time in history, people aged 65 years and more in the world will outnumber children aged younger than 5 years (UN, 2014). With populations ageing across the EU and the world, pensions, healthcare and long-term care systems risk becoming financially unsustainable, as a shrinking labour force may no longer be able to provide for a growing number of older people.

Healthy ageing, like active ageing, emphasizes the need for public actions across multiple sectors and enabling older people to remain a resource to their families, communities and economies. This concept is object of the European Commission's policy directed towards 'helping people stay in charge of their own lives for as long as possible as they age and, where possible, to contribute to the economy and society'.

The European innovation partnership (EIP) on active and healthy ageing was created in 2011 and aims to foster innovation that will promote active ageing and raise healthy life expectancy. Later, the United Nations General Assembly declared 2021–2030 the Decade of Healthy Ageing and asked WHO to lead the implementation. The Decade of Healthy Ageing is a global collaboration bringing together governments, civil society, international agencies, professionals, academia, the media and the private sector for 10 years of concerted, catalytic and collaborative action to foster longer and healthier lives.

The Decade builds on the WHO Global Strategy and Action Plan and the United Nations Madrid International Plan of Action on Ageing and supports the realization of the United Nations Agenda 2030 on Sustainable Development and the Sustainable Development Goals. The Decade of Healthy Ageing (2021–2030) seeks to reduce health inequities and improve the lives of older people, their families and communities through collective action in four areas: changing how we think, feel and act towards age and ageism; developing communities in ways that foster the abilities of older people; delivering person-center integrated care and primary health services responsive to older people; and providing older people who need it with access to quality long-term care (WHO).

To answer to these new challenges, the Ministry of Health has also drawn up simple practical rules that can be followed to ensure healthy ageing (Ministero della Salute, 2012):

1. New dietary strategies: vary the diet as much as possible, alternate meat with fish and legumes, take fibres and abundant amounts of fresh vegetable and fruit, limit the use of salt and prefer olive oil, moderate the intake of sweets and coffee and drink at least a litre and a half of water a day. Use of new functional foods. In case of specific pathologies, follow the diet prescribed by a medical professional.

2. Stay active and on movement: physical activity is useful for the body and for the mind; it is enough a walk a day of at least 30 minutes at moderate speed.

3. Take care of interests and relationships: maintain friendships and relationships with family members, undertake new relationships and take care of some interest such as volunteering.

4. Living in a safe home: improve the lighting of interior spaces, install fire prevention systems, overhaul the electrical system and wear comfortable shoes.

5. Attention for the seasons that are too hot or too cold: avoid going out during the hottest hours in the summer season, drinking a lot and wearing light clothing; during the winter, cover themselves properly, take meals and hot drinks and avoid staying too close to stoves or others heat sources.

6. Contact the family doctor if there are any health problems.

Healthy ageing is not only an urgent topic for the public health, but also the ultimate goal of ageing research; the mechanisms of healthy ageing, in fact, have been widely investigated and are still object of study through a wide spectrum of disciplines, including molecular biology, neurology and public health. All the achievements in this field could be useful to develop new valid prevention strategies (Kennedy et al., 2014).

1.3.3 Nutritional strategies for healthy ageing

Nutrition is recognised as one of the major determinants of successful ageing and it is one of the best prevention strategies (Krondl et al., 2008).

The effect of diet on human health has been amply reported in many epidemiological, population based, and randomised clinical trials, providing evidence that nutrients and other substances obtained from a wide variety of foods promote health, maintain metabolic homeostasis, and reduce the incidence of disease. However, the optimal dietary strategy to prevent chronic degenerative diseases remains a challenging and a highly relevant issue. Different types of diet were imposed to public attention, but the one that got the greatest interest is certainly the Mediterranean diet that has been extensively reported to be associated with a favourable health outcome and a better quality of life (Sofi et al., 2013; 2014). Increasing evidence suggests that a Mediterranean style diet that is high in fruit, vegetables, fish and with the adequate dietary protein intake may reduce the risk of mild cognitive impairment, Alzheimer's disease and support lean body mass maintenance (Shlisky et al., 2017). This is good evidence, since the percentage of malnourished elderly people is going to increase, and with it also the number of cases

of “nutritional frailty” (Salazar et al., 2017). Therefore, nutritional strategies in the elderly should be addressed considering their intestinal microbiota, the immune system as well as nutritional deficiencies and needs as a whole (Louis and Flint, 2009).

A relevant nutritional strategy for well-being is the use of functional foods. The concept of functional foods was first introduced in Japan in the mid-1980s when the Japanese government began funding research programs to study the ability of certain foods to influence physiological functions and as a convenient solution to prevent/cure some of the chronic health problems (Ohama et al., 2006). Along the time, the definition of functional food has been revisited, until the definition at the international conference in 2014: *“Natural or processed foods that contains known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease”* (Martirosyan and Singh, 2015). In accordance with this definition, “functional foods” are foods or food ingredients that provide additional health benefits behind the basic nutritional needs, due to their bioactive compounds that act synergistically, contributing to improve the quality of life and longevity (Gibson et al., 2017; Martirosyan and Singh, 2015).

Studying the biological and cellular mechanisms responsible for the ageing, it is possible to select conventional functional foods or develop modified/fortified functional foods with promising profiles. Some examples are foods with antioxidant activity, that have received attention because of their potential role in modulating oxidative stress associated with ageing and chronic conditions. Resveratrol is a natural compound found in several plants which has been shown to have powerful antioxidant properties with the ability to neutralize free radicals. Resveratrol is present in red wine, like other flavonoids, and it has been indicated as one of the potential protective factors in the prevention and treatment of a large number of diseases, included cardiovascular health, blood glucose control, skin health, bone health, and memory (Honari et al., 2019). Relevant to the potential benefits of resveratrol is how different foods/supplements affect the gut microbiota, especially those promoting increased growth of beneficial microbes in the gut. Resveratrol and other polyphenols have been suggested to inhibit pathogenic bacteria while stimulating the growth of beneficial bacteria, exerting prebiotic-like effects. Importantly, recent publications show that some of the effects of resveratrol

including reduced atherosclerosis and improved glucose homeostasis (Walker et al., 2018; Gal et al., 2021). Moreover, dietary components of foods containing antioxidant activity such as vitamin E and C or specific forms of fatty acids such as (n-3) polyunsaturated fatty acids (PUFA) been largely studied. Higher levels of vitamin A and E were found in human healthy centenarians, reinforcing the antioxidant–life span relationship. While Vitamin C is a promising anti-hypertensive, once its plasmatic levels were inversely associated with arterial blood pressure (Bischoff-Ferrari et al., 2004). Fruits and vegetables are considered functional foods, as they have specific compounds that have been shown to prevent and reduce diseases. These compounds are known as phytonutrients (lycopene, anthocyanin, beta-carotene, lutein, zeaxanthin, isothiocyanate and allicin). Fruits also provide fibres, vitamins, minerals, flavonoids and terpenes, many of which provide protection against oxidative processes (Ohama et al., 2006). Vegetables are also an important source of phytosterols, the intake of which is associated with a reduction in serum cholesterol levels and of cardiovascular risk. Extra Virgin Olive Oil (EVOO) and its bioactive components (fatty acids, phytochemicals, such as polyphenolic compounds, squalene and a-tocopherol) have shown a broad range of promising activities in different inflammatory and autoimmune diseases (Masala et al., 2007). The foods just described are only a part of the wide variety of functional foods that are continuously studied and characterized by researchers as useful support for health and well-being.

1.3.4 Probiotic functional foods for elderly people

The gastrointestinal tract and its resident microbes have far-reaching implications for health. When elderly population is increasing worldwide, the analysis of the contribution of the microbiota to healthy ageing assumed greater significance, especially from the perspective of improving the wellness of aged people by modulating the gut microbiota (Salazar et al., 2017).

Among functional foods, probiotics enriched foods may exert positive effects on the composition of gut microbiota and on some age-related problems (Salazar et al., 2013).

Probiotic concept, from the Greek “pro-bios” (for life), was coined by Metchnikoff, even if Parker, in 1974, first defined probiotics as: “organisms and substances which contribute to intestinal microbial balance” (Caramia and Silvi, 2011). Further studies brought to the most appropriate definition formulated by FAO/WHO (2002): “Live

microorganisms which, when administered in adequate amount, confer a health benefit to the host".

The addition of probiotics to food to confer health benefits has become widespread in recent years, garnering interest from the scientific community and the general public with a probiotic market that has expanded rapidly (Power et al., 2014). The microbial species used in the probiotic industry must respect some specific criteria. Bacteria should be safe for human use (reference to the criteria adopted by EFSA), not be pathogenic, toxic, mutagenic, or carcinogenic in the host organism and be genetically stable without a plasmid transfer mechanism, especially concerning antibiotic resistance (Granato et al., 2010). Also, the amount is important, the adequate numbers of viable cells (therapeutic minimum) need to be consumed regularly. The minimum effective dose, which affects the intestinal environment and provides beneficial effects on human health, is considered to be 10^6-10^9 live microbial cells a day. In addition to survive the stomach and arrive to the intestine in optimal numbers, probiotic strains must be able to adhere to intestinal epithelium and/or mucus, persist and multiply in the gut to maintain its metabolic activity and confer their probiotic properties in the human body (Linares et al., 2017).

Probiotics administration in old people have been documented to increase intestinal levels of beneficial lactobacilli, bifidobacteria, and enterococci, with reduced levels of enterobacteria.

This may well counter the age-related changes in the intestinal microbiota and therefore prevent diseases such as *Cl. difficile*-associated diarrhoea, constipation, and common respiratory and gastrointestinal infections. These preventative effects against the risk of infections have been attributed partly to the immune-modulatory and anti-inflammatory activities of probiotics by increasing NK (natural killer) cell activity, increased phagocytosis, which may underline the improvement of the immune-ageing (Nagpal et al., 2018).

The administration of probiotics in the elderly has proved useful also to promote intestinal well-being in terms of reduced orofecal transit time, decreased abdominal pain, bloating, the number of loose stools, and diarrhoea episodes (Ouwehand et al., 2014).

Probiotic bacteria may influence the intestinal microbiota and/or induce beneficial host responses with different mechanisms: production of antimicrobial compounds (e.g. bacteriocins), the reduction of luminal pH through the production of SCFAs, competition

with pathogens for nutrients and prebiotics, competitive exclusion of pathogens for adhesion to epithelial cells, production of growth substrates (e.g. vitamins, SCFAs and exopolysaccharide), enhanced intestinal barrier function (e.g. increased mucus and b-defensin secretion and/or modulation of cytoskeletal and tight junction protein phosphorylation), modulation of immune response.

It is important to take into consideration that the mechanism of action of probiotics is strain specific, so different probiotic strains have been associated with different effects related to their specific capacities (Power et al., 2014). A variety of genera and species of microorganisms are considered as potential probiotics. *Lactobacillus* spp. and *Bifidobacterium* spp. are the most common bacteria used by the food industry, as they are typically granted "Generally recognised as safe" (GRAS) status by several regulatory agencies and they are also species dominant inhabitants in the human intestine. However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Propionibacterium* and *Saccharomyces* yeasts (e.g., *S. cerevisiae* and *S. boulardii*) and filamentous fungi (e.g., *Aspergillus oryzae*) are also used as probiotics due to their health promoting effects (Tripathi *et al*., 2014). Most of the cited strains are lactic acid bacteria (LAB), found in traditional fermented food and used in food fermentation-controlled process. The most known is the *Lactobacillus acidophilus* that is also considered the predominant lactobacillus in the intestinal tract of heathy humans (Rivera-Espinoza and Gallardo-Navarro, 2010).

Probiotics and their effects on human health have been demonstrated both within different food matrices and as single or mixed microbial culture preparation (Verdenelli et al., 2009; Silvi et al., 2014). Dairy-based products are suggested to be the main carriers for the delivery of probiotics: yogurts, fresh cheeses, fermented milks, ice cream, baby food, milk powder, frozen dairy desserts, whey-based beverages, sour cream, buttermilk, normal and flavoured liquid milk (Coman et al., 2013). Among dairy products, yogurt has been used for a long time and has been known to maintain human health by its excellent nutrition profile. Yogurt is accessible and convenient to consume by the older population.

Furthermore, also synbiotic products, such as the synbiotic yogurt have been studied. The term "synbiotic" refers to the combined relationship between probiotics and prebiotics.

In general, the potential health effects of synbiotic products on elderly include changes in the composition and activity of the intestinal microbiota, especially by promoting the growth of *Bifidobacterium* and *Lactobacillus* species in the gut. Some age-related conditions and discomforts are improved, such as evacuation frequency (constipation/diarrhoea), immunomodulation, nutrient availability, especially regarding increased calcium and magnesium availability (Bedani et al., 2016). For example, with ageing the intestinal motility may change because of the decrease in *Bifidobacterium* in the intestinal microbiota. Studies demonstrated that fruit juice supplemented with *L. rhamnosus* and *Propionibacterium freudenreichii* resulted in a 24% increase in defecation frequency; moreover, milk fermented with *L. casei shirota* in Parkinson's Disease (PD) patients with constipation resulted in a significant improve of stools with a normal consistency, and significant reductions in bloating, abdominal pain, and the feeling of incomplete emptying (Martínez-Martínez et al., 2017).

1.4 PROJECT OVERVIEW

The research activities subject of the current thesis work are part of the project "Assessment of the effect of a diet based on probiotic functional food on low-grade inflammation in healthy senior subjects: PROBIOSENIOR project" financed by the Marche Region within the call: "Promoting innovative solutions to address local community challenges in the area of health and well-being" - POR MARCHE FESR 2014-2020. This study, coordinated by Synbiotec Srl (Camerino, Italy), received the favourable opinion of the Regional Ethical Committee (Comitato Etico Regione Marche-CERM). The companies involved, besides the University of Camerino, are the University of Florence, Fidoka Srl (MC), and the functional foods producers: dairy farm Val d'Apsa in Urbino (PU), Synbiofood Srl in Civitanova Marche (MC) and Synbiotec Srl in Camerino (MC).

PROBIOSENIOR was an *in vivo*, double-blind, randomized, placebo-controlled trial. The study design, developed into three phases, is based on the administration of a dietary supplementation with functional foods and/or dietary supplements, containing probiotic microorganisms, to elderly people, and monitor any changes. The main aim of the study is the evaluation of the effects of a probiotic functional food-based diet on the reduction of low-grade inflammation, in healthy senior subjects.

1.5 OUTLINE OF THE THESIS

The objectives of the thesis were: evaluation of the reduction of low-grade inflammation in elderly people and their well-being status after probiotic supplementation. This wide purpose actually includes further aspects and evaluations, before and after the intervention: assessment of the composition of the intestinal microbiota and the integrity of the barrier function; monitoring and follow-up of physical and psychological well-being; monitoring of the level of biomarkers of inflammation, such as the high sensitivity C-reactive protein (hsCRP), and blood clinical parameters; evaluation of immunological status, through the modulation of the expression of some circulating factors, the nutritional status, intestinal health status, metabolomics, information on general health status.

The content of each chapter are summarized in below: **Chapter 2 –** PROBIOSENIOR project: a probiotic functional foods-based diet to improve healthy ageing; **Chapter 3 –** Questionnaires as effective monitoring of health status in the elderly; **Chapter 4 –** Assessment of probiotics effects on gut microbiota modulation in the elderly; **Chapter 5 –** Probiotics effects on short-chain fatty acids production by gut microbiota; **Chapter 6 –** Monitoring of biogenic amines on probiotics-supplemented seniors; **Chapter 7 –** Effects of probiotic supplementation on inflammatory markers in the elderly; **Chapter 8 –** Impact of probiotic supplementation on hsCRP concentration and haematological parameters; **Chapter 9 –** Interactive nutritional education interventions for elderly people to promote healthful ageing; **Chapter 10 –** Final conclusions.

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CHAPTER II

PROBIOSENIOR PROJECT: A PROBIOTIC FUNCTIONAL FOODS-BASED DIET TO IMPROVE HEALTHY AGEING

2.1 ABSTRACT

In Italy senior population is growing, as well as in many western countries. The population of older people (65 years or more) in the EU-27 will increase significantly, rising from 90.5 million at the beginning of 2019 to reach 129.8 million by 2050. During this period, the number of people in the EU-27 aged 75-84 years is projected to expand by 56.1%, while the number aged 65-74 years is projected to increase by 16.6% (EUROSTAT, 2019). This demographic change will most likely lead to an increase of elderly people with age-related diseases. The challenge of modern societies is to study and develop new strategies and public actions to decrease morbidity, slowing down the ageing process and increasing the number of healthy seniors. To improve health status and general well-being, nutrition and functional foods play a key role. PROBIOSENIOR is a research project that aims to evaluate the effects of a probiotic functional food-based diet on the reduction of low-grade inflammation in elderly subjects, promoting healthy ageing. PROBIOSENIOR is a randomised, double-blind, placebo-controlled human trial that recruited elderly healthy volunteers for the administration of specific functional foods containing the probiotic mixture SYNBIO® for 3 or 6 months. Lifestyle and eating habits information, psychological well-being and nutritional status data were collected through the administration of specific questionnaires, while biological parameters were analysed by collecting faecal, urine and blood samples. Questionnaires and biological samples were administered and/or collected at baseline and after the dietary intervention, to monitor all changes occurred. The biological parameters evaluated, related to inflammation status and well-being, were high sensitivity C-reactive protein (hsCRP), cytokines and other inflammatory biomarkers, haematological parameters, composition and diversity of gut microbiota, Short-Chain Fatty Acids (SCFAs), and biogenic amines.

2.2 INTRODUCTION

The number of older people in Europe is expected to increase from 17% in 2010 to approximately 30% in 2060; this trend reflects the future of Italy very much, which is in fact becoming an “old country” (EUROSTAT, 2019). If this change has a positive aspect that is the extension of life expectancy, on the other hand, having an old population means the increase of chronic degenerative diseases, morbidity, disability, and health care needs. For this reason, understand which the ageing mechanisms and study new strategies are public health priorities. Strong evidence indicate that older organisms tend to develop a pro-inflammatory status that is characterized by high levels of pro-inflammatory markers in cells and tissues. Some relevant biological markers are C-reactive protein (CRP), interleukin-6 and interleukin-1 that are considered predictors of physical and cognitive performance among elders. CRP levels increase physiologically with ageing, and it plays a key role in inflammatory and disease processes and host responses to infection (Velissaris et al., 2017). This low-grade inflammation status, called inflammageing, is a risk factor for age-associated chronic diseases, cardiovascular disease (CVD), and other negative health outcomes (Ferrucci and Fabbri, 2018). Potential mechanisms of inflammageing include genetic susceptibility, central obesity, increased gut permeability, changes of microbiota composition, cellular senescence, oxidative stress, immune cell dysregulation, and chronic infections (Ferrucci and Fabbri, 2018). Inflammageing, through changes in lifestyle and age-related modifications in intestinal physiology, profoundly also affects the homeostasis of intestinal microbiota (Zapata and Quagliarello, 2015). The composition and abundance of gut microbiota change continuously until adulthood, and it reflects lifestyle, geographic, racial, and individual differences. Although most of these changes appear to be harmless, a major shift in the gut microbiota composition (dysbiosis) can trigger harmful local and systemic inflammation (Ragonnaud and Biragyn, 2021). Recent reports indicate that dysbiosis is increased in ageing, and elderly gut microbiota is enriched in pro-inflammatory commensals at the expense of beneficial microbes. Dysbiosis seems to be one of the primary causes of ageing-associated morbidities; certainly, it triggers a chain of pathological and inflammatory events. Some examples are the alteration of levels of microbiota-affected metabolites, impaired function and integrity of the gastrointestinal

tract, and increased gut leakiness (Ragonnaud and Biragyn, 2021). The change of some key members of intestinal microbiota is also associated with the depletion of specific Short Chain Fatty Acids (SCFAs) in the GI tract. Since one of the functions of SCFAs is the regulation of the immune function and the maintenance of the functionality of GI epithelial barrier, their reduction can involve nutritional and immunological effects, contributing to the health impairment of older people (Mäkivuokko et al., 2010).

Among all variable factors, lifestyle and diet are shown to be essential in the control of human healthy ageing, and thus, longevity. Basing on this evidence, during PROBIOSENIOR project we aimed to understand and exploit the impact of diet and nutritional strategies on general and intestinal well-being of elderly. The project used innovative functional foods and nutraceuticals containing probiotics to improve parameters related with ageing mechanisms, such as immune status, intestinal microbiota, SCFA, and haematological profile. Considering the increasing percentage of people affected by functional disorders and other difficulties, PROBIOSENIOR's strategy represents an easily applicable action that will respond to the new challenges of modern societies, with a positive impact on the social-health system.

2.3 MATERIALS AND METHODS

2.3.1 Study design

PROBIOSENIOR was an *in vivo*, randomised, double-blind, placebo-controlled study, in which healthy elderly subjects (65-85 years) from boarding homes or private houses in Marche region (Italy)were recruited for the administration of probiotic-enriched foods or dietary supplements to assess the effect on the health status. This project was based on a primary hypothesis that a functional food-based diet decreases the low-grade inflammation and on a secondary hypothesis based on the balance of the intestinal microbiota, strengthening of the immune system, increased intestinal well-being and improvement of the general quality of life. The experimental phase developed 3 phases:

I. Subjects recruitment and enrolment.
II. Dietary intervention.
III. Wash-out.

All subjects recruited according to the inclusion criteria explained in the following paragraphs, were randomly divided into two parallel groups: the probiotic-supplemented group (foods or alternative capsule) and the placebo group. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and national laws and regulations. The study protocol was approved by the Ethics Committee (CERM, Marche, Italy) and the participants gave written informed consent for the enrolment.

2.3.2 Intervention structures

The experimental intervention was developed in three different areas of Marche Region (Italy), where subjects living in boarding homes or in their private houses were recruited.

The boarding homes involved in this project belong to different Aree Territoriali Sociali (ATS) of the region:

1. ATS 16 - “P. Burocchi”, Penna San Giovanni; “L’Immacolata”, Sant’Angelo in Pontano; “Ex Opera Pia Bonfranceschi”, Loro Piceno (MC);
2. ATS 17 - “ASP Lazzarelli”, San Severino Marche; “A. Chierichetti”, Gagliole (MC).
3. Other volunteers have been recruited in private houses in Camerino (MC) and in nursing homes/long-term hospitalization facilities “Villa Igea” in Ancona.

2.3.3 Preliminary activities

One of the preliminary activities is the project presentation to the structures. Meetings with the responsible of boarding homes and the staff involved, such as workers, residents of the boarding home and their relatives or caregivers have been fundamental to explain all the aspects of the study project: protocol, project phases, expected objectives and any questions, doubts and curiosities. Together with the nursing staff, potential subjects have been selected for each structure, basing on the inclusion and exclusion criteria specified in the clinical protocol of the project. To ensure a better knowledge of the project, a brochure summarising the various phases and goals of the treatment was distributed among the participants (Figure 1 and Annex 1).

Figure 1. PROBIOSENIOR project brochure distributed to boarding home guests.

2.3.4 Enrolment of volunteers and randomization

The recruitment and enrolment of subjects were carried out by the doctor in charge of the study. Eligible participants were all healthy adults aged 65-85 years, that meet the inclusion and exclusion criteria of the clinical protocol. Inclusion and exclusion criteria were meant to ensure patient safety during the study, provide data of subject appropriateness, to minimize withdrawal and ensure that primary endpoints of the study were reached. The inclusion criteria usually describe the main characteristics of the target population and the actual sub-group of subjects. The inclusion criteria of this study were:

- healthy persons (chronic diseases, controlled with proper medications were accepted);
- age;
- acceptance of study protocol.

The exclusion criteria aim to prevent the enrolment of subjects that could interfere/disturb/confuse the quality of the data/study. The exclusion criteria were:

- the use of probiotics continuously, in the previous two months;
- use of antibiotics in the previous month;
- chemotherapy treatments;
- severe respiratory, hepatic and/or renal insufficiency;
- use of anti-inflammatories in the previous four months;

- malnutrition: BMI <18.5 kg / m2 or weight loss> 10% in the last 6 months.

There were also criteria for leaving the study:

- non-continuation of administration;
- severe illness;
- continued use of antibiotics and/or laxatives or other probiotics;
- inappropriate enrolment;
- adverse event;
- personal motivations: a patient may withdraw from the study at any time and for any reason.

2.3.5 Foods preparation for dietary intervention

The functional foods proposed by the involved food companies, and then supplemented to the volunteers, were six: “ricotta” and “primo sale” cheese (produced by Caseificio Val d’Apsa, Urbino), yogurt, “mozzarella” cheese and fruit juice (produced by Synbiofood srl, Civitanova Marche), and chocolate bar (produced by local chocolate maker).

All the food products were enriched with the SYNBIO® mixture, supplied by Synbiotec Srl (Camerino, Italy). SYNBIO® is a 1:1 combination of two probiotic bacterial strains, *Lactiplantibacillus rhamnosus* IMC 501® and *Lactiplantibacillus paracasei* IMC 502®, at the concentration of 10^9 live cells per gram (Verdenelli et al., 2009, 2011; Silvi et al., 2014). The addition of SYNBIO® was done during the food usual production process, directly on production site following the specific technology and procedures to enrich each single kind of food (Verdenelli et al., 2009). The functional foods were also subjected to microbiological analysis (shelf-life and vitality of probiotics tests) and organoleptic properties analysis. As alternative to foods, the dietary supplement consisted of capsules containing the same SYNBIO® mixture at a concentration of 15 billion live cells per gram, always provided by Synbiotec Srl (Silvi et al., 2014). Probiotic functional foods and their placebo version, as well as for dietary supplements, were packaged identically in appearance.

2.3.6 Dietary intervention

The dietary intervention was based on the administration of probiotic functional foods for a 12-weeks or 24-weeks intervention period. Volunteers are called to consume one functional food or capsule a day, in order to assume the appropriate amount of probiotic bacterial cells (Silvi et al., 2014). In agreement with the staff of the structures, the most suitable probiotic functional foods were selected for the dietary intervention. The choice of all/or some of those proposed foods obviously took into account the dietary habits and taste of the subjects, but also specific personal needs (diabetes, dysphagia and other difficulties). The foods were provided periodically directly to the structures (for all months of intervention), respecting the shelf-life of each product and the indication of consumption. For the blind supplementation, each probiotic functional food/placebo were labelled only with the name and the identification code of the participants.

2.3.7 Collection and analysis of samples

According to the project protocol, after the enrolment of subjects, questionnaires were administered and biological samples collected.

Personal data collection and information regarding the health status were collected from the volunteer and/or with the help of the nursing staff, through interviewers, face-to-face, using questionnaires uploaded to a dedicated platform, developed for the project. The help of a nursing staff/health assistant, depending on the patient's health status (e.g., individuals with cognitive impairments not able to answer some questions) was accepted.

Like questionnaires, all biological samples were collected at baseline, to understand the health status of subjects, and after 6-months of dietary supplementation, to assess all changes occurred after the intervention. Blood samples were collected and immediately delivered to the analytic laboratory for the analysis, while faecal samples and urines were collected into plastic containers and frozen at -20°C until the analysis.

2.3.8 Statistical analysis

All parameters were measured in duplicate, and data are expressed as mean ± standard deviation. The statistical significance of the differences obtained between different sampling timepoints was evaluated using the Student's *t* test and ANOVA test. Statistical significance was considered when the probability value $P < 0.05$.

2.4 RESULTS

2.4.1 Enrolment of volunteers and randomization

Volunteers were recruited in boarding homes and private houses belonging to the different Aree Territoriali Sociali (ATS) described in the previous paragraph. Two hundred and thirty (230) participants were assessed for eligibility (Figure 2), and a total number of 206 participants met the including criteria. For the allocation of the subjects into the two different supplemented groups (probiotic and placebo), a computer-generated list of random numbers was used.

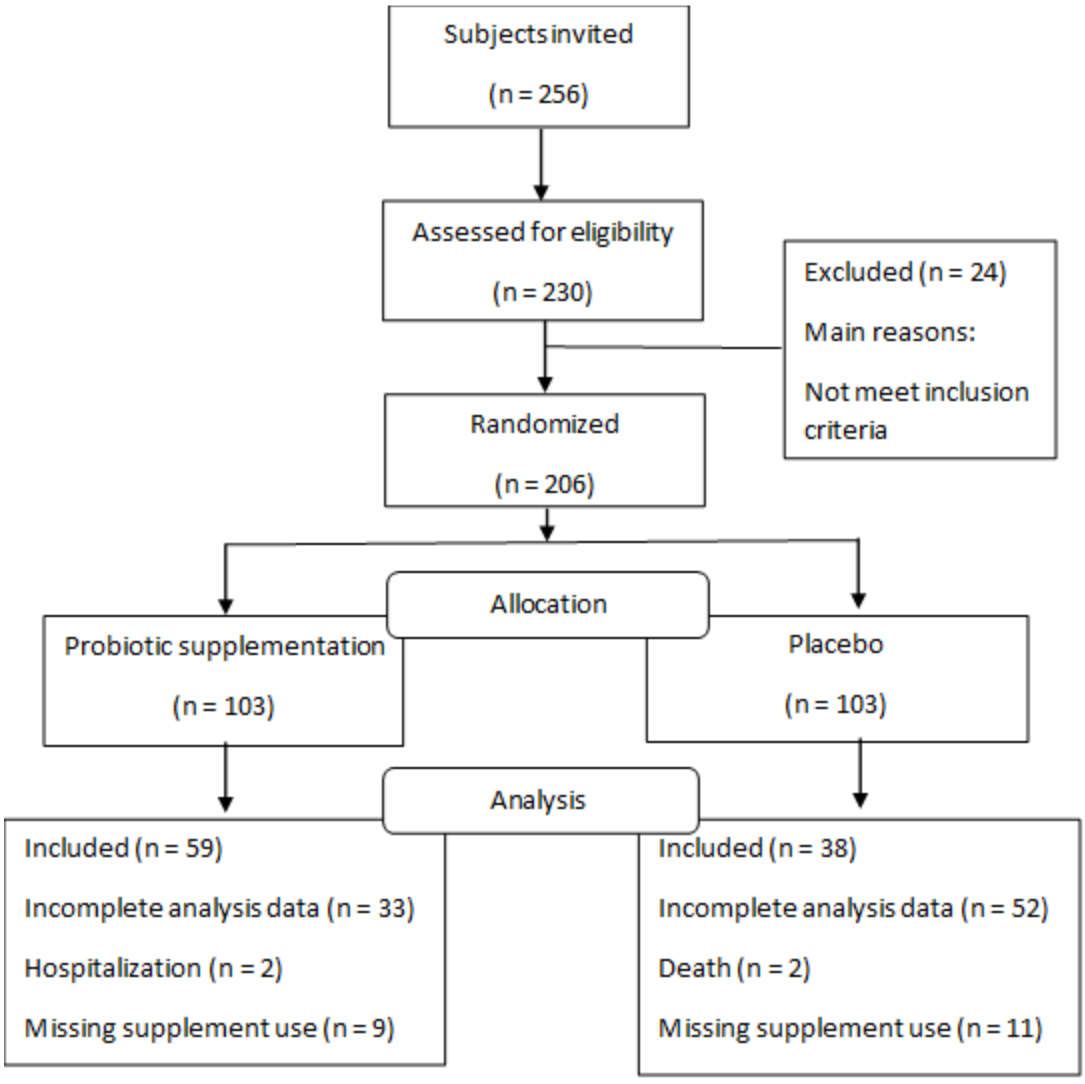

Figure 2. Flowchart of participants.

2.4.2 Dietary intervention

Dietary intervention started approximately one week after the collection of the first biological samples. Among the six different types of proposed functional foods, chocolate bar (sugar free - dark chocolate), yogurt and fruit juice (orange and pomegranate juice) were the most appreciated foods selected for the seniors of the boarding homes (Figure 3).

For people living in their private house, Fìdoka Srl (San Ginesio, Itay), in collaboration with the ICT team Unicam (University of Camerino, Camerino, Italy), designed and developed an innovative system, called Smart Box. It is a box to store and weekly deliver foods/supplements to the enrolled free-living subjects, with the purpose to also monitor the compliance in eating the functional foods every day. This system in fact was connected to a specific software which records the corresponding data (Figure 4).

Figure 3. Probiotic functional foods: chocolate bar, yogurt and fruit juice; Probiotic dietary supplement: capsule.

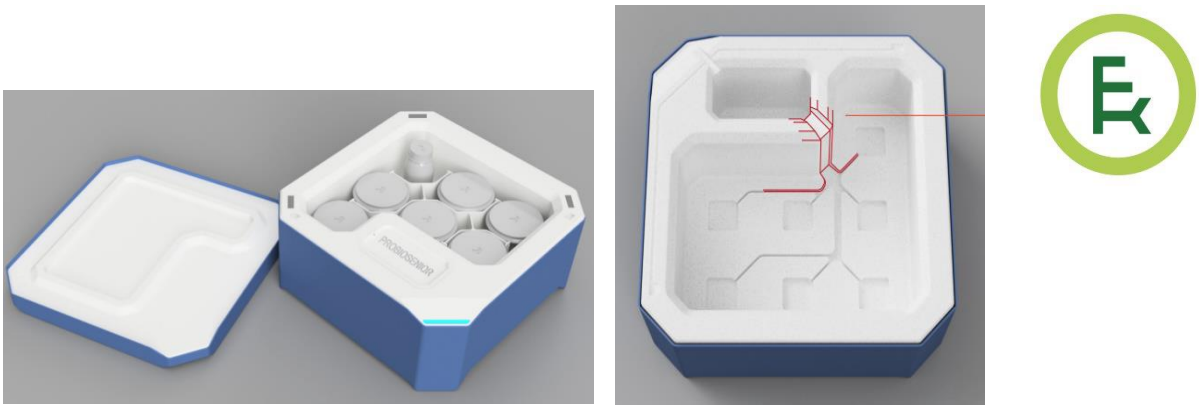

Figure 4. The Smart Box, designed by Fìdoka Srl (San Ginesio, Italy).

2.4.3 Collection and analysis of samples

The questionnaires of the present study were compiled with the purpose to know and record the daily habits and the general well-being of subjects under study, while biological samples were collected to assess their health conditions at baseline and after the dietary intervention.

The primary expected outcome of the study was a decrease in plasma concentration of high sensitivity C-reactive protein (hsCRP).

Secondary outcome measures included:

- Evaluation of the immunological state, through determination of the modulation of the expression of cytokines and inflammatory biomarkers in the serum.
- Evaluation of haematological parameters, such as glucose, albumin, haemoglobin, haematocrit, total cholesterol, low- and high-density lipoproteins, and triglycerides.
- Changes in the composition of the gut microbiota, evaluated by quantification with Real-Time PCR of selected bacterial groups present at the faecal level; microbial diversity studied by 16S metagenomic analysis.
- Metabolomic analysis by determination of the concentration of Short-Chain Fatty Acids (SCFA) in faecal water samples using gas chromatography techniques.
- Determination and quantification of eleven biogenic amines (histamine, serotonin, dopamine, epinephrine, putrescine, cadaverine, spermine, spermidine, tyramine, tryptamine, phenylethylamine) in human urine through high performance liquid chromatography (HPLC) coupled with a Fluorescence method (FLD).
- Changes in nutritional status, evaluated through Mini Nutritional Assessment (MNA) questionnaire.
- Changes in the quality of life, evaluated through the questionnaire PGWBI (Psychological General Well-Being Index).
- Changes in the nutritional habits, evaluated through Lifestyle and eating habits questionnaire.
- Changes in intestinal function, evaluated in accordance with the Bristol Stool Chart.

- Evaluation of the physio-pathological status, using Pathological anamnesis and Pharmacological therapy questionnaire.

2.5 DISCUSSION AND CONCLUSIONS

In Italy senior population is growing worldwide. The demographic change leads to study and develop new strategies and public actions to decrease the incidence of age-related diseases and morbidity, promoting the healthful ageing. To improve health status and general well-being, nutrition and functional foods represent a crucial aspect. Current advances in the gut microbiota field open new frontiers for functional foods, nutraceuticals, and probiotics. Indeed, in recent years, interest in this area of study has continued to increase, since the new technologies and applications offer the possibility to improve the scientific knowledge on the dietary and health benefits of these interventions. PROBIOSENIOR is a research project that aims to evaluate the effects of a probiotic functional food-based diet on the reduction of low-grade inflammation in elderly subjects. PROBIOSENIOR is a randomised, double-blind, placebo-controlled human trial that involved 206 clinically healthy volunteers who met the including criteria of the study. Selected functional foods containing the probiotic mixture SYNBIO® were administered for 3 or 6 months. Questionnaires were proposed before and after the dietary intervention; the same timepoints were used for monitoring the biological and biochemical parameters. The first outcomes were hsCRP, as recognized marker of low-grade inflammation, and modulation of GUT microbiota. In conclusion, we explored the effects of probiotic functional foods on age-related inflammation and general well-being of elderly, with the purpose to improve their daily consumption to ameliorate the quality of life of older adults.

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CHAPTER III

QUESTIONNAIRES AS EFFECTIVE MONITORING OF HEALTH STATUS IN THE ELDERLY

3.1 ABSTRACT

Twenty-first century societies are in continuous development and are facing daily challenges. Since the improved living conditions and life expectancy, elderly people are increasing in number. Therefore, the evaluation and monitoring over time of the well-being and health status of the population, especially of elderly, become of central importance for the modern societies (Kalache and Keller, 2001).

Among the multidimensional tools to investigate a community, questionnaires are useful instruments to describe the population in all its aspects. Our study examined the physical and psychological health profile of an elderly community before and after a dietary intervention, in which we used probiotic functional foods and their placebo version. The purpose was to evaluate if the supplementation had some effects on the elderly status. The questionnaires used to evaluate all the variables and aspects of the community were: Lifestyle and eating habits questionnaire, Pathological anamnesis and Pharmacological therapy questionnaire, Mini Nutritional Assessment (MNA), The Psychological General Well-Being Index (PGWBI) and Bristol Stool Chart. The MNA questionnaire showed most people at risk of malnutrition at baseline; after the experimental phase (T1), slight improvements in nutritional status were observed in both groups. At the same time, an evident impairment was recorded in placebo-supplemented subjects (61.5% *vs* 37.5% in probiotic group). The PGWBI questionnaire described a population with moderate distress at baseline; at T1, a general improvement was observed, but the amelioration was higher in probiotic group (80% of cases) than in placebo one (50%). Finally, the lifestyle and eating habits, and the pathological anamnesis and pharmacological therapy monitoring shows, as expected, no fundamental changes. No valid and reliable results were collected through the Bristol Stool Chart, because of the difficulties to harvest data. In conclusion, despite the limitations of the human trial and the complexity of biological pathways, our results suggest the apparent benefits of

probiotic supplementation on psychological well-being and nutritional status, improving the quality of life in elderly. However, further investigation is necessary to confirm these preliminary findings and define the responsible mechanisms.

3.2 INTRODUCTION

Quality of life and well-being have recently been recognized as the central purpose of health care. However, the concept of quality of life is wide and not always defined uniformly. This concept is essentially subjective and it includes several items and variables to take into account (Haraldstad et al., 2019). Therefore, it is much more difficult to evaluate these aspects in elderly people life, because of a wide range of diseases and age-related pathologies (Terada et al., 2002). The questionnaire could be one of the most valid tools to assess some life aspects of elderly people, especially before a human trial (Kostecka and Bojanowska, 2021). Basing on the topics and questions developed, questionnaires are multidimensional tools that can detect important aspects such as lifestyle and eating habits, nutritional status, regularity and health of intestinal tract, pathological anamnesis and relative pharmacological therapy, and the psychological general well-being status. The administration of one or more questionnaires allows to define a more accurate profile of the single elderly subject or of the entire community under study. Therefore, our current aims were to describe the health profile, in all its aspects, of the entire population under study at baseline. The same evaluation was repeated after the dietary intervention, to compare the results and assess all changes occurred.

3.3 MATERIALS AND METHODS

3.3.1 Questionnaires administration

Questionnaires have been compiled by a nurse in charge and directly uploaded to the project platform (designed and supplied by Innovative Technology Srl., Camerino); if necessary, subjects were helped by assistants. The purpose of compiling questionnaires was to record and get to know the nutritional status of subjects, their eating habits and lifestyle, their anamnestic and pharmacological data, psychological well-being and the intestinal well-being and regularity. All data collected before and after dietary intervention were used to investigate about a possible relationship between the well-being of the subjects and the administration of functional foods.

3.3.2 Lifestyle and eating habits questionnaire

The lifestyle and eating habits questionnaire (Annex 2) was settled up by project partner "Cooperativa Sociale Onlus" (COOSS) of the Marche Region, with the Ethic Committee approval. This questionnaire was used to evaluate the subject's habits in order to choose the best nutritional supplementation strategies for them. This, in accordance with the aim to integrate the functional foods within own diet, without changing nutritional habits and respecting the tastes of the person. Moreover, investigate nutritional habits could be useful to evaluate a possible correlation between nutritional habits and sample's parameters (e.g., composition of the gut microbiota), given the high impact of the lifestyle and the diet on the health status of the subject.

The first group of questions was related to age, weight (Kg), height (m), waist circumference (cm). Another group of questions was about lifestyle, such as the living situation, physical activity, use of dietary supplements. The last and most numerous questions were about the eating habits: the type of followed diet, the presence of allergies/intolerances, the quantity of daily meals and their composition (frequency in the consumption of pasta, rice or pizza, bread and derivatives, dairy products, legumes, eggs, meat, fish, processed meats, fruits and vegetables) and daily water or other drinks consumption.

3.3.3 Pathological anamnesis and Pharmacological therapy questionnaire

The pathological anamnesis and the pharmacological therapy questionnaires (Annex 3) were designed up by the "COOPERATIVA SOCIALE MARCHE ONLUS" (COOSS), with Ethic Committee approval. This questionnaire allowed the collection of information about pathological status of the subjects. In particular, it provided information on the presence of lipid and glucose metabolism disorders, respiratory, skeletal, genitourinary, gastrointestinal, and nervous system disorders, past surgical intervention, family history of cardiovascular diseases, psychological disorders, past nutritional rehabilitation, degree of self-sufficiency, current or past substances and alcohol abuse, and the use of drugs. The purpose of the questionnaire was to confirm the eligibility of recruited subjects and to assess, at the end of the study, whether the presence of diseases or the use of drugs can behave as confounders in the analysis.

3.3.4 Mini Nutritional Assessment (MNA)

The Mini Nutritional Assessment (MNA) (Annex 4) was designed by Nestlé company to provide a single, rapid assessment of nutritional status of elderly subjects (65 years of age and older). The MNA is a screening tool for identifying malnutrition or the risk of malnutrition; it usually provides a quick and easy medical support to detect malnourished subjects or those at risk of malnutrition in hospitals or communities (Bollwein and Volkert, 2013).

The MNA test was composed of simple measurements and brief questions:

- anthropometric measurements (weight, height, and weight loss);
- global assessment (six questions related to lifestyle, medication, and mobility);
- a dietary questionnaire (eight questions, related to the number of meals, food and fluid intake, and autonomy of feeding);
- subjective assessment (self-perception of health and nutrition).

The questions were organized in 18 items (A-R), where each letter corresponds to each question, and they were rated with a score from 0 to 3; each item has been divided into subgroups. The first group of questions A-F were the screening part, with a subtotal maximum screening score of 14 points. A score of 12 or greater indicated the person was well nourished and needed no further intervention. A score of 8-11 indicated the person was at risk of malnutrition. A score of 7 or less indicated the person was malnourished. For additional information on factors that may impact nutritional status, there were questions G-R called assessment, with a maximum of 16 points. At the end, the scores from assessment and screening parts were added to calculate a total assessment (maximum 30 points) that was the overall malnutrition index.

The MNA score distinguished between elderly subjects with adequate nutritional status, MNA ≥ 24; malnourished, MNA < 17; at risk for malnutrition, MNA between 17 and 23.5 (Bollwein and Volkert, 2013).

3.3.5 The Psychological General Well-Being Index (PGWBI)

The Psychological General Well-Being index (PGWBI) (Annex 5) was a validated measure of Health-related Quality of Life (HRQoL), widely used in clinical practice and epidemiological research to provide a general subjective assessment of the intrapersonal affective or emotional states, the psychological well-being and, thus, the health (Grossi et

al., 2006). The questionnaire (Annex 5) consisted of 22 items and six domains: anxiety, depression, general wellbeing, vitality, positivity and self-control. Each item had six possible scores (from 0 to 5) and each domain had a minimum of 3 and a maximum of 5 items. The sum of the scores for all domains gave the well-being index, which reached a theoretical maximum of 110 points, representing the best achievable level of well-being (Grossi et al., 2006). This questionnaire was administered through an interview, only to subjects able to answer (e.g., considering the presence of subjects with mental disorders). Moreover, PGWBI was repeated at the end of the study with the aim to evaluate potential changes in the quality of life.

3.3.6 Bristol Stool Chart

The Bristol Stool Chart is a 7-point scale used in clinical and research practice for stool form measurement, created to classify the shape and texture of human faeces into categories. Stool consistency is a central component in the description of normal or altered bowel habit. This scale is useful for evaluating some pathologies (irritable bowel syndrome), the gut transit time (GTT), IBS-D or chronic constipation, and in general the intestinal functionality (Blake et al., 2016).

Figure 1 is a pictorial representation of each stool type, classified as:

Type 1: Separate hard lumps (hard to pass)

Type 2: Lumpy, sausage-shaped

Type 3: Sausage-shaped with cracks on the surface

Type 4: Sausage-shaped or snake-like; smooth and soft

Type 5: Soft blobs with clear-cut edges (easy to pass)

Type 6: Fluffy pieces with ragged edges; mushy

Type 7: Entirely liquid, watery, no solid pieces

Stools that were well-formed and easy to pass (Types 3 and 4) were considered "ideal". Stools that were hard and difficult to pass (Types 1 and 2) indicated constipation. Stools that contained excess liquid or were entirely liquid indicates diarrhoea (Types 5, 6, and 7) (Blake et al., 2016).

Figure 1. Bristol Stool Chart: stool shapes and precise descriptions regarding form and consistency.

3.4 RESULTS

3.4.1 Questionnaires outcomes at baseline

3.4.1.1 Lifestyle and eating habits

Thanks to the Lifestyle and eating habits questionnaire, we collected information not only about the daily life habits of enrolled subjects, but also a general description of the population under study.

Unfortunately, the data collected are not available for all the subjects recruited, due to the difficulties during the compilation or due to particular situations. Below, then, will be reported only the data extrapolated from the completed questionnaires.

Of people who filled the questionnaires, 72 out of 108 were female, while 36 males. 77 of 108 were subjects living in boarding homes and 31 in private houses. As reported in the Table 1, people belong to different age ranges: 65-70, 70-75, 75-80, and over 80 years old. The Body Mass Index (BMI) was also considered, only 63 volunteers were available. BMI is a measure of nutritional status and body composition, that tend to change with ageing. Consequently, the changes in body composition associated with ageing may result in BMI increase of 1.5-2.5 kg/m^2 in both men and women, even when body weight remains constant (Babiarczyk and Turbiarz, 2012). Of the studied population, free-living subjects had a BMI score > 23. While 15% of female in boarding homes had a score

between 19-21, 5% of female and 12% of male between 21-23 and 80% of female and 88% of male had a score >23 (Table 1).

Table 1. Population characteristics related to age, gender and BMI.

SUBJECTS IN BOARDING HOMES				**SUBJECTS LIVING IN PRIVATE HOUSES**			
FEMALE	66%	***MALE***	34%	***FEMALE***	68%	***MALE***	32%
AGE RANGE	**%**	***AGE RANGE***	**%**	***AGE RANGE***	**%**	***AGE RANGE***	**%**
65-70	13	65-70	28	**65-70**	24	65-70	22
70-75	10	70-75	8	**70-75**	12	70-75	44
75-80	23	75-80	32	**75-80**	12	75-80	22
>80	55	>80	32	**>80**	53	>80	11
BMI	**%**	***BMI***	**%**	***BMI***	**%**	***BMI***	**%**
≤19	0	≤19	0	**≤19**	0	≤19	0
19-21	15	19-21	0	**19-21**	0	19-21	0
21-23	5	21-23	12	**21-23**	0	21-23	0
≥23	80	≥23	88	**≥23**	100	≥23	100

The second part of questionnaire concerned lifestyle topics, such as physical activity, lunch/dinner out, consumption of vitamin or mineral supplements, dietary supplements or food bars and medicines. From the results, explained in detail in the Table 2, 52% of people practises physical activity, while 48% of them does not. Type of activity and frequencies are different, but the main “sport” is walking. Most subjects have 3 meals a day (87%), 51% of people uses to have lunch or dinner out, but with low frequency, 1 o 2 times per month. Hardly anyone (4%) reported to take vitamin or mineral supplements, dietary supplements, or food bars (5%). All the subjects reported to take drugs, and the 82% takes more than 3 drugs a day. In addition, some of them reported difficulty to remember hiring schedules. Subjects who don’t follow specific diets are 79%, while low-glucose, low-sugar, and gluten free diets are followed by 20% of people. Finally, just 3% of enrolled subjects is intolerant or allergic (Table 2).

Table 2. Lifestyle questionnaire, the values are expressed in percentage basing on the replied questions.

PHYSICAL ACTIVITY (%)		FREQUENCY (%)		TYPE OF ACTIVITY (%)	
YES	52	**EVERY DAY**	31	**WALKING**	46
NO	48	**1-3 TIMES/WEEK**	18	**GYMNASTICS**	3
		RARELY	12	**CYCLETTE**	2

MEALS/DAY (%)		LUNCH OR DINNER OUT (%)	
4 meals	1	YES	51
3 meals	87	NO	47
2 meals	11	**FREQUENCY (%)**	
1 meal	2	1-2 TIMES/MONTH	29
		RARELY	32

	VITAMIN OR MINERAL SUPPLEMENTS (%)	DIETARY SUPPLEMENTS AND FOOD BARS (%)	DRUGS (%)	MORE THAN 3 DRUGS/DAY (%)	PARTICULAR DIET (%)	INTOLERANCE OR ALLERGY (%)
YES	4	5	99	82	20	3
NO	21	81	1	20	79	97

Figure 2 shows the results from eating habits questionnaires. Most subjects had pasta/rice/pizza (76.9%) and also bread/breadsticks/crackers (81.3%) every day of the week, both for lunch and dinner. All subjects had milk, yogurt and fresh cheeses, for breakfast, lunch or dinner. 28.6% of people consumes dairy products every day, especially milk or yogurt, while the frequency of cheese consumption was 2 or 3 days per week by 58.2% of population under study. Legumes and eggs, and the based-meat products, were consumed 2-3 times a week, for lunch or dinner by most subjects, respectively 71% and 79%. Meat or fish were consumed all days of the week by 25.3% of people, 4-6 days per week by 18.7% and 2-3 days per week by 86.8%, both for lunch and dinner. All subjects had cakes or candies, the majority (42.9%) with a frequency of 2-3 days a week, as a snack, with a preference for packaged biscuits/snacks. All the results are reported in Figure 2.

About daily eating habits, fruits and vegetables are an important source of vitamins and minerals, especially for elderly people. All people consumed these two categories every day, with the frequency of 1 or 2 times (Figure 3). Figure 3 also shows the water consumption expressed as glasses in a day. 50% of subjects had from 3 to 5 glasses of water (alternatively tea), 47.7% took more than 5 glasses and just 2.3% of population took

less than 3 glasses, daily (Figure 3). Other drinks consumed were fruit juices, and thickened water, in case of dysphagia condition.

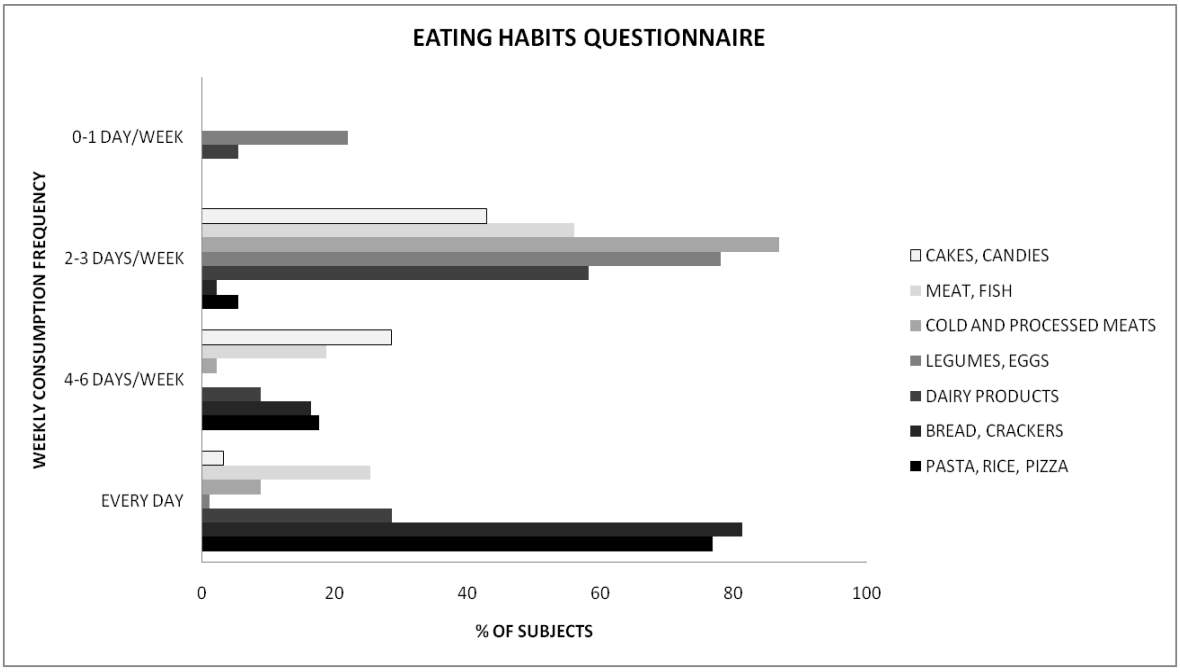


Figure 2. Eating habits questionnaire, results expressed as percentage of subjects related to the consumption frequency of foods categories.

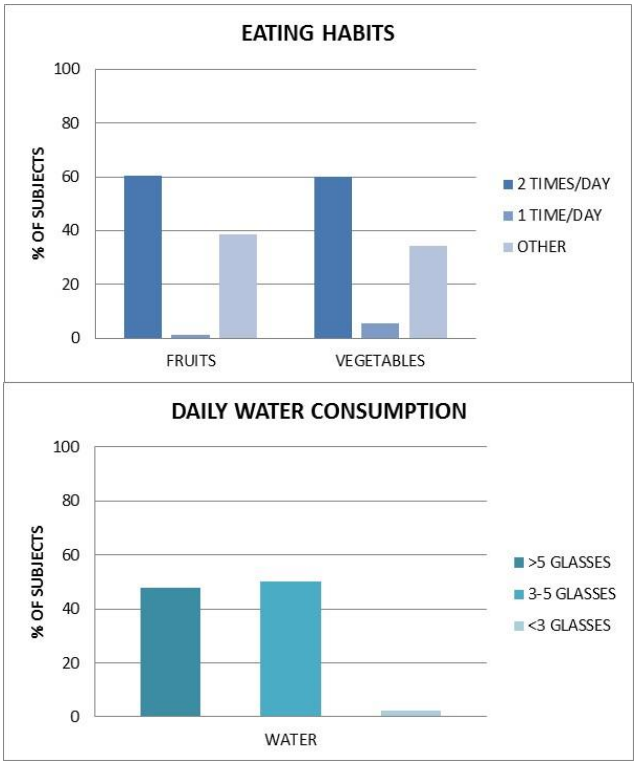


Figure 3. Eating habits questionnaire, results expressed as percentage of subjects related to the daily consumption frequency of fruit, vegetables, and water.

3.4.1.2 Pathological anamnesis and Pharmacological therapy

The results of pathological anamnesis and Pharmacological therapy questionnaire are shown in Table 3. The enrolled people who joined the questionnaire, answering all the questions were 86. Pathologies have been divided into eight clusters of diseases regarding the same apparatus or system or on the basis of similar symptoms.

Thirty-one subjects had high plasma cholesterol levels and 18 out of 86 had high plasma lipid levels; 20 people had glucose blood levels higher than 116 mg/ml and the same number had diabetes or took insulin.

Considering the diseases of the cardiovascular system, 51 out of 86 subjects reported arterial hypertension or to take antihypertensive drugs, 37 reported atherosclerotic diseases and 12 suffered of cardiac insufficiency.

In relation to respiratory apparatus diseases, only 4 of the 86 subjects reported apnoea during sleep (OSAS) or restrictive respiratory failure (COPD), and 7 subjects referred wheezing during moderate exertion or snoring.

Arthrosis, already documented with radiography or other investigation, was reported by 35 elderly volunteers as skeletal apparatus disorders. Some subjects referred to use a walking aid, in particular 14 used the stick, 15 the walker aids, and 21 the wheelchair; moreover only 33 out of total 86 people were completely self-sufficient.

For what concern the genitourinary system, 34 subjects had stress incontinence.

Seven declared to have fat liver, gallbladder stones, gastritis or reflux.

Twelve subjects had Parkinson's disease among the nervous system pathologies and 3 out of total volunteers suffered of seizure.

Only 9 subjects had surgery experiences on different apparatus.

Behaviour disorders such as major depressive disorder, bipolar and schizophrenic disorder, borderline and antisocial personality disorders, panic and obsessive-compulsive disorders, were certified by a physician, psychotherapist and/or psychiatrist for a total of 28 subjects out of 86. Only 1 person reported a past abuse of wine or other alcoholic beverages.

Only 4 and 3 subjects referred respectively stress eating and eating disorders (bulimia, binge eating) (Table 3).

All of the enrolled subjects used more than two drugs per day, the most common were to treat lipid and glucose metabolic disorders, cardiovascular system diseases and skeleton-muscular problems.

Table 3. Pathological anamnesis questionnaire, results are expressed as absolute frequency (sample size n=86).

		YES	NO	OTHER
ALTERATION OF LIPID METABOLISM	High cholesterolemia or to take antidyslipidemic therapy	31	55	
	High triglyceridemia or to take antidyslipidemic therapy	18	68	
ALTERATION OF GLYCEMIC COMPENSATION	High blood sugar (above 116 mg)	20	66	
	Diabetes and/or to take oral therapy or insulin	20	66	
DISEASES OF CARDIOVASCULAR SYSTEM	High blood pressure or to take antihypertensive therapy	51	35	
	Atherosclerotic disease (ischemic heart disease, angina, heart attack, stroke, etc.)	37	49	
	Cardiac insufficiency (breathlessness, sweating) during acts of daily life or at rest.	12	74	
RESPIRATORY APPARATUS	Sleep apnoea (OSAS) or restrictive respiratory failure (COPD), documented with spirometry/Cardiorespiratory monitoring /polysomnography	4	82	
	Wheezing during moderate effort or snoring	7	79	
SKELETAL APPARATUS	Arthrosis (Hip, knees, spine) already documented with X-ray or other investigation	35	51	
	To use walking aid: A- stick B-walker C-wheelchair D-no	A-14 B-15 C-21	D-36	
GENITOURINARY APPARATUS	Polycystic ovary	0	86	
	Stress Incontinence (e.g. Urine Loss During Stress or Coughing)	34	52	
GASTROENTERIC APPARATUS	Fatty liver, calculi of the cholecystic, gastritis, reflux	7	79	
NEUROLOGICAL APPARATUS	Parkinson	12	74	
	Epilepsy	3	83	
SURGERY EXPERIENCE	Have you had surgery?	9	59	19
		YES: Removal of gallbladder part of intestine (2016), femur breast, appendicectomy, hip surgery, inguinal hernia, cervical hernia, hysterectomy, hernia, knee		
FAMILY HISTORY	Family history of premature cardiovascular disease (myocardial infarction, stroke and/or sudden death before the age of 55 for the father or 65 for the mother)	11	75	
BEHAVIOUR	Major depressive disorder, bipolar disorder, schizophrenic disorder, borderline and antisocial personality disorders, panic and obsessive-compulsive disorder, certified by a doctor psychotherapist and/or psychiatrist	28	58	
	Eating disorders (stress eating)	4	82	
	Eating disorders such as bulimia nervosa, compulsive nutrition, night nutrition certified by a doctor psychotherapist and/or psychiatrist	3	83	

OTHERS	Failed 3 or more outpatient weight loss programs?	1	85	
	Have you been admitted to the Nutritional Rehabilitation Unit in the past?	3	82	1
	Are you self-sufficient?	33	18 (7 personal care needs)	21
	Need oxygen therapy and/or respirator?	4	84	
	Current or past substance use	0	79	8
	Abuse of wine or other alcoholic beverages (e.g., more than 3 glasses per day)?	1	85	

3.4.1.3 Mini Nutritional Assessment (MNA)

The MNA questionnaire was used to evaluate the nutritional status of elderly volunteers, by the sum of scores of each answer. The total MNA score distinguishes three different categories: 24, out of 85 subjects who joined the questionnaire, resulted in an adequate nutritional status, MNA ≥ 24; 15 subjects resulted malnourished, MNA < 17; the remaining 46 subjects resulted to be at risk for malnutrition, 17 < MNA < 23.5. The MNA scores as mean values (± standard deviation) obtained for each nutritional status is reported in Table 4.

Table 4. Mini Nutritional Assessment results.

MINI NUTRITIONAL ASSESSMENT			
	TOTAL MNA SCORE	**MEAN±S.D.**	**n. of subjects**
MALNOURISHED	**<17**	14±2	**15 (85)**
AT RISK OF MALNUTRITION	**17-23.5**	21.1±1.6	**46 (85)**
NORMAL NUTRITONAL STATUS	**>24**	25.8±1.3	**24 (85)**

3.4.1.4 The Psychological General Well-Being Index (PGWBI)

The PGWBI questionnaire was compiled by 64 subjects. The PGWBI was calculated following a default scheme proposed by Mario Negri Institute (Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy) and accessible online: for each subject, the completed questionnaire was uploaded on the online default scheme, together with gender and age information of the person.

For each uploaded questionnaire, the system elaborated a score related to six domains and a total score. The domains are anxiety, depression, general wellbeing and self-control, vitality and positively; each of them has a maximum row points number. Each domain's score was then converted into a 0-100 scale, used as reference score for the elaboration of results. Figure 4 shows the mean scores obtained from all subjects for each domain. Considering the results proposed by the used default system and calculated on the base of a standard Italian elderly population, the values had been considered as follows: PGWBI < 60: severe distress, 60-73: moderate distress, 74-97: no distress, > 97: well-being. Table 5 represents the mean values of each domain but also the PGWB Index of the total population.

Considering that the lowest PGWB Index obtained was 34.7 points and the highest was 93.0, the population had a PGWBI Index, calculated as mean value, of 63.3, corresponding to a moderate distress psychological state, in line with that one of an elderly community.

Table 5. Scores (0-100 scale) obtained for each domain of singular subjects, and PGWB Index. The scores are expressed as mean ± SD.

ANXIETY	DEPRESSION	GENERAL WELL-BEING	VITALITY	POSITIVITY	SELF-CONTROL	PGWB Index
71.9±15.3	74.2±16.6	60.8±18.8	59.1±19.1	52±18.4	62.1±18.8	63.3±14.3

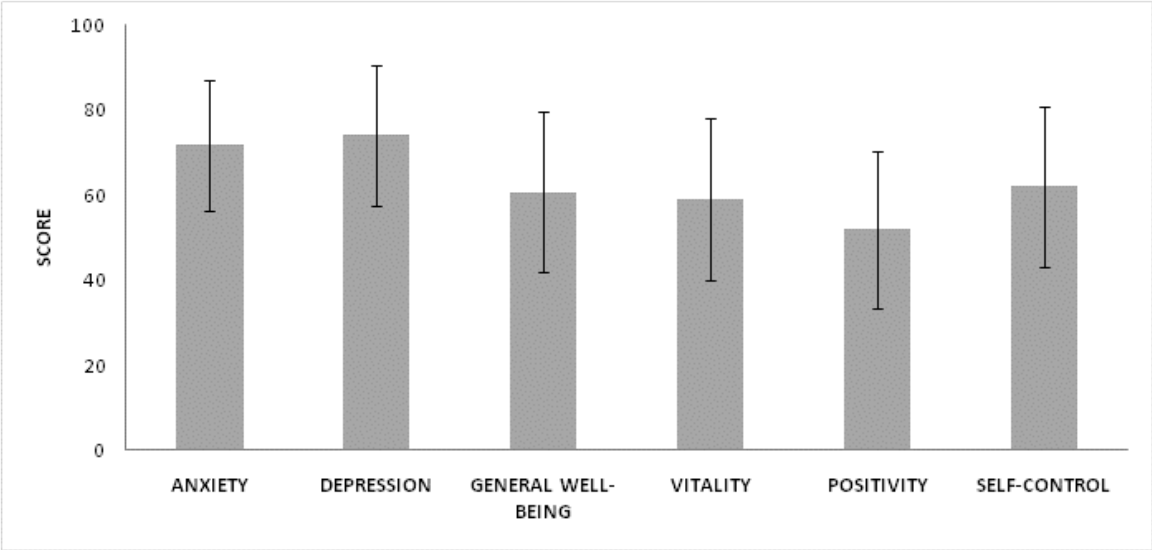


Figure 4. Scores obtained for each domain of PGWBI referred to the total population under study.

3.4.1.5 Bristol Stool Chart

The collection of a constant and accurate stool description from volunteers and boarding home operators resulted challenging, and very often impossible. Although this parameter was not taken into account for the purpose of the study, due to lack of data, I report the few data collected about 22 subjects before the dietary intervention. People who recorded types 1 and 2 stools were respectively 3 and 4. Six subjects had type 3 stools, well-formed and easy to pass, that indicate a good intestinal health. Three and 6 subjects out of 22 recorded types 5 and 6 stools, containing excess liquid or entirely liquid. Nobody referred type 4 and 7 stools.

3.4.2 Questionnaires outcomes after the intervention

3.4.2.1 Lifestyle and eating habits

The Lifestyle and eating habits questionnaire was compiled after the dietary intervention by only 39 subjects, 26 of which received the probiotic functional foods and 13 had the placebo supplementation. Following an accurate analysis of the questionnaires, no relevant changes were evidenced before and after the dietary intervention. Regardless of the type of supplementation and the structure of residence, all enrolled subjects maintained in general the same lifestyle and eating habits.

3.4.2.2 Pathological anamnesis and Pharmacological therapy

The Pathological anamnesis and Pharmacological therapy questionnaire was compiled by 37 subjects after the dietary intervention. Of 37 subjects living in boarding homes and in private houses, 24 received the probiotic functional foods and 13 had the placebo supplementation. The pathological conditions of the population remained stable and unchanged after the dietary intervention respect to baseline, as well as pharmacological therapy, in all groups under study.

3.4.2.3 Mini Nutritional Assessment (MNA)

The Mini Nutritional Assessment questionnaire was chosen as the evaluation method to assess the nutritional status changes of subjects. The MNA questionnaires were compiled by 37 subjects after experimental phase. To compare the data before and after the dietary intervention, having more realistic feedback, I selected just the questionnaires of which I had the corresponding one compiled at baseline. Of 37 subjects, 24 received the probiotic functional foods and 13 had the placebo supplementation; most of them had the supplementation for 6 months, with the exception of 5 people (of probiotic-supplemented group). Table 6 is referred to the mean scores of MNA for each group, considering the type of supplementation, at baseline (T0) and after intervention (T1). As reported in Table 6, in the probiotic-supplemented group, the percentage of malnourished subjects positively decreased after supplementation from 16.7% to 12.5%; the percentage of elderly at risk of malnutrition increased and the number of people with normal nutritional status slightly decreased. The placebo-supplemented group, less numerous, presented the same number of malnourished subjects before and after the intervention, a decreased percentage of people at risk of malnutrition and an improvement of those in a normal nutritional status.

Table 6. Mini Nutritional Assessment at baseline (T0) and after the dietary intervention (T1), referred to the different type of supplementation. Results expressed as mean ± SD. and relative percentage.

MINI NUTRITIONAL ASSESSMENT (screening score)									
		PROBIOTIC T0	**%**	**PROBIOTIC T1**	**%**	**PLACEBO T0**	**%**	**PLACEBO T1**	**%**
MALNOURISHED	**<17**	14.9±1.0	16.7	15.7±0.8	12.5	15.3±0.4	15.4	14±1.4	15.4
AT RISK OF MALNUTRITION	**17-23.5**	22.2±1.0	37.5	21.5±1.3	45.8	20.6±1.6	53.8	19.8±1.3	46.2
NORMAL NUTR. STATUS	**>24**	26.3±1.3	45.8	26.2±1.2	41.7	25.3±1.0	30.8	24.3±0.4	38.5

Further elaboration of data was performed in collaboration with Prof. De Leone team in our University. The graphs reported are related to the results collected: first we compared the total scores, relative to the initial time T0 and the final time T1; subsequently specific clusters were considered to better explain the development of the data.

From the results of the MNA questionnaire of all subjects, shown in Figure 5, significant changes in nutritional status are not observed: most people involved continue to be at risk of malnutrition, regardless of the type of supplement, while only few subjects (5 out of 37) passed to a better status. Figures 6 and 7 report the results analysing the type of supplementation. 37.5% of subjects in probiotic group maintained the same nutritional status, respect to 7.7% in placebo group. Moreover, an evident but not significant impairment was observed in placebo-supplemented subjects with a decline in the nutritional status in 61.5% of cases, respect to 37.5% in probiotic group. On the contrary, the rate of improvement in nutritional status was similar between the two groups, with the 25% and 30.8% of cases after probiotic and placebo supplementation, respectively. Taking into account the gender, a better nutritional status was noticed in males than females, regardless the supplementation (data not shown); the same result was observable already at T0, so it is not attributable to an effectiveness of treatment. Further evaluations were done considering the time of supplementation, the 6-month intervention seemed to be more effective than a shorter one (3 months), despite the small sample size undermines the reliability of the results obtained (data not shown).

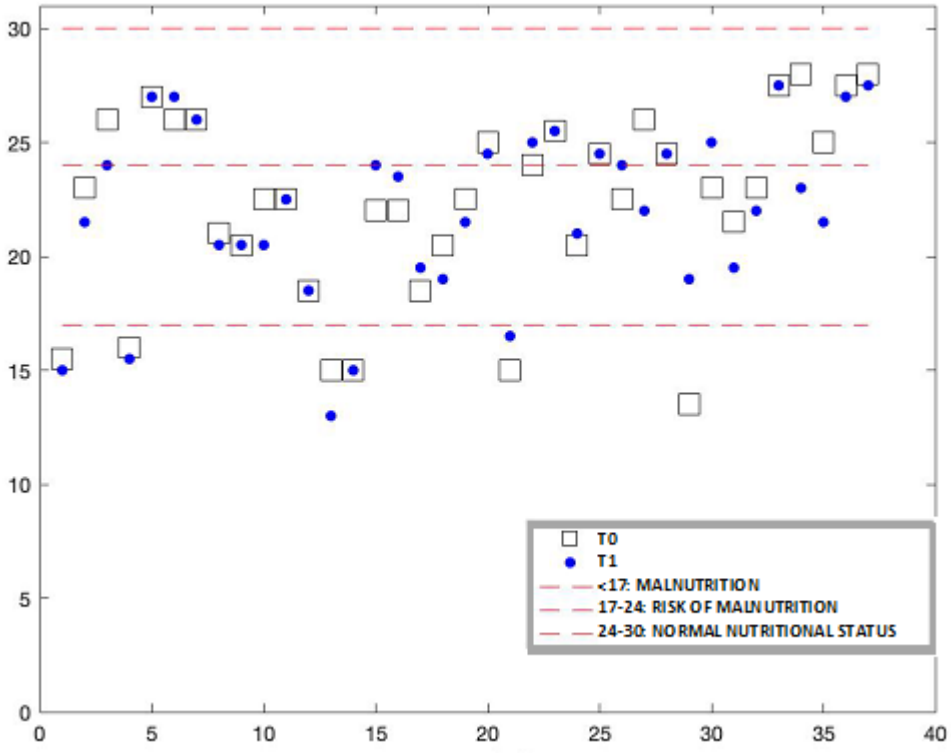
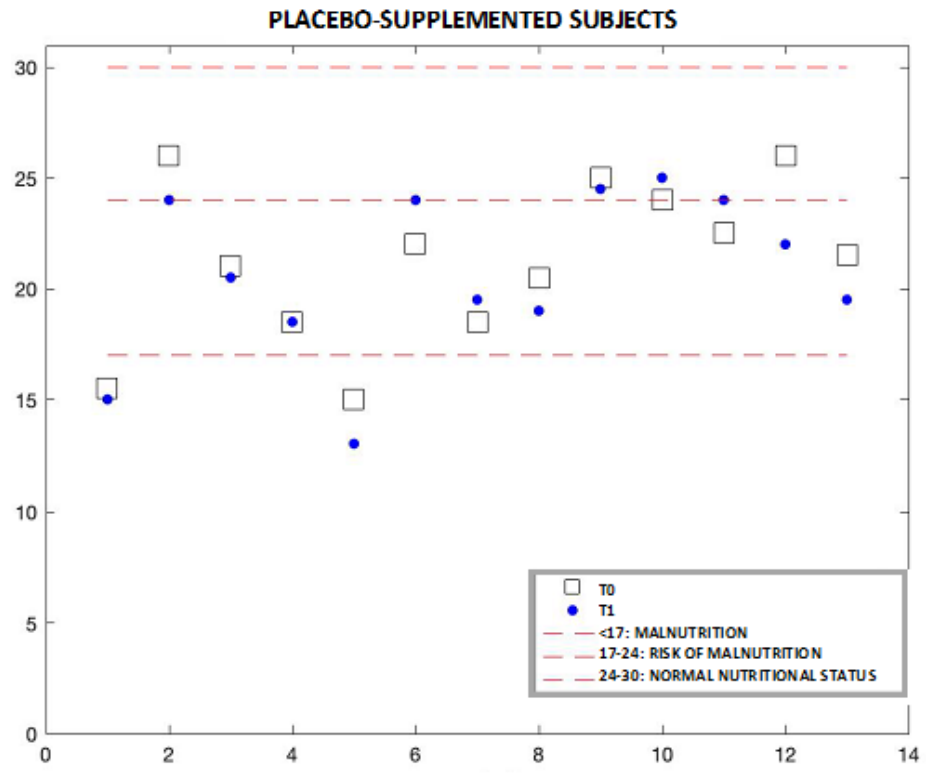
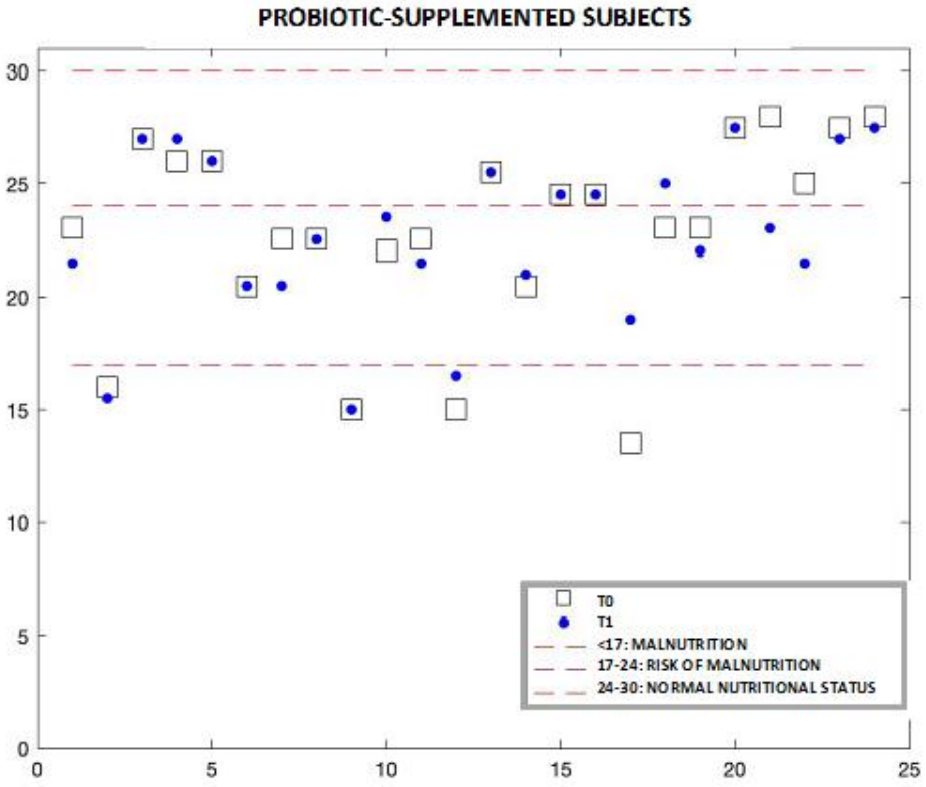


Figure 5. Comparison of MNA results at T0 and T1 of all subjects.





Figures 6 and 7. Comparison of MNA at T0 and T1 of all subjects, basing on the type of supplementation.

3.4.2.4 The Psychological General Well-Being Index (PGWBI)

The PGWBI questionnaire was used as a method of evaluation for changes in the quality of life and in the general well-being of subjects. Below are reported the results collected at baseline time point (T0) and after the dietary intervention (T1); in total 25 subjects, 15 of which received the probiotic supplementation and 10 had the placebo diet. Just 2 of them received the supplementation for less than 6-months period.

The variables used to organize all data are duration of supplementation, type of supplementation, and gender.

Table 7 put in evidence that, for both groups, there was an improvement in the psychological well-being, indicated by an increased PGWB Index mean score. Since the positive improvement is referred to both probiotic and placebo groups it doesn't have a direct correlation with the effectiveness of treatment. Figure 8 shows all the changes into each domain of the PGWBI questionnaire. Although the total score of general psychological status improved in both groups, analysing the single domains, the changes trend is variable. The probiotic supplementation seems to influence general well-being and vitality, while after placebo intervention, depression and self-control had a better score than baseline.

Table 7. Scores (0-100 scale) of each group and time point, for each domain and PGWBI index. The scores are expressed as mean ± SD.

	ANXIETY	DEPRESSION	GENERAL WELL-BEING	VITALITY	POSITIVITY	SELF-CONTROL	PGWB Index
PRO T0	77.1±14.1	73.8±16.6	58.5±15.0	56.7±15.9	57.3±17.5	63.7±14.0	64.5±10.7
PRO T1	73.1±10.6	78.3±6.8	68.1±14.2	67.3±14.7	58.7±14.3	68.7±17	69.0±10.7
PLA T0	71.2±21.2	67.0±18.4	67.7±20.1	61.0±18.7	49.5±24.3	61.5±20.2	63.0±17.9
PLA T1	71.6±16.5	78.2±14.0	72.5±17.5	66.5±15.1	54.0±17.8	70.8±17.4	68.9±14.0

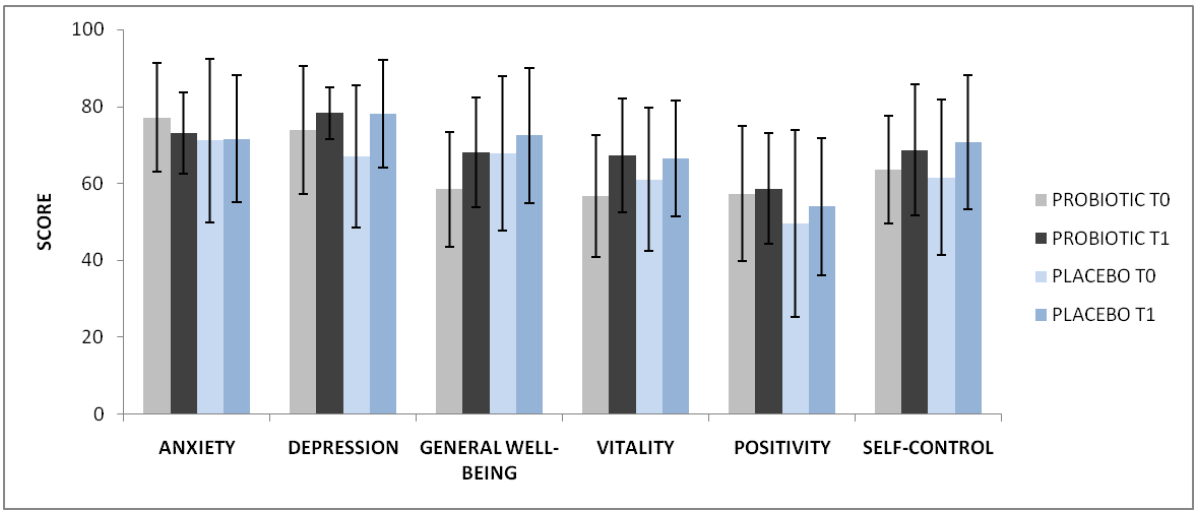


Figure 8. Scores obtained for each domain referred to different time points and supplementation.

Figures 9-13 show all changes after the intervention, comparing the results obtained at different time points. In each graph, the dashed horizontal lines identify 4 bands, useful for the interpretation of the score of the questionnaire. Going up, the first corresponds to a state of severe distress, the second to a state of moderate distress, the third to a state without distress and the last to a state of positive well-being.

Figure 9, which shows the results of the PGWBI questionnaire of all population, it was observed an increase in the PGWB index in 68% of cases, regardless the type of supplementation. In addition, 20% of subjects worsened their status, while 12% of them maintained it unchanged. A further elaboration was done taking into account the gender, but not significant differences were observed between females and males (Figures 10 and 11). However, the subdivision according to the type of supplementation, shown in Figures 12 and 13, suggests that there are interesting differences between the subjects treated with probiotic and those treated with placebo. Despite the sample size difference, an important improvement (80% of cases) was recorded in probiotic group respect to placebo one (50% of cases). Moreover, 30% of subjects evidenced a worsening in PGWB index after placebo supplementation and only 13.3% after probiotics.

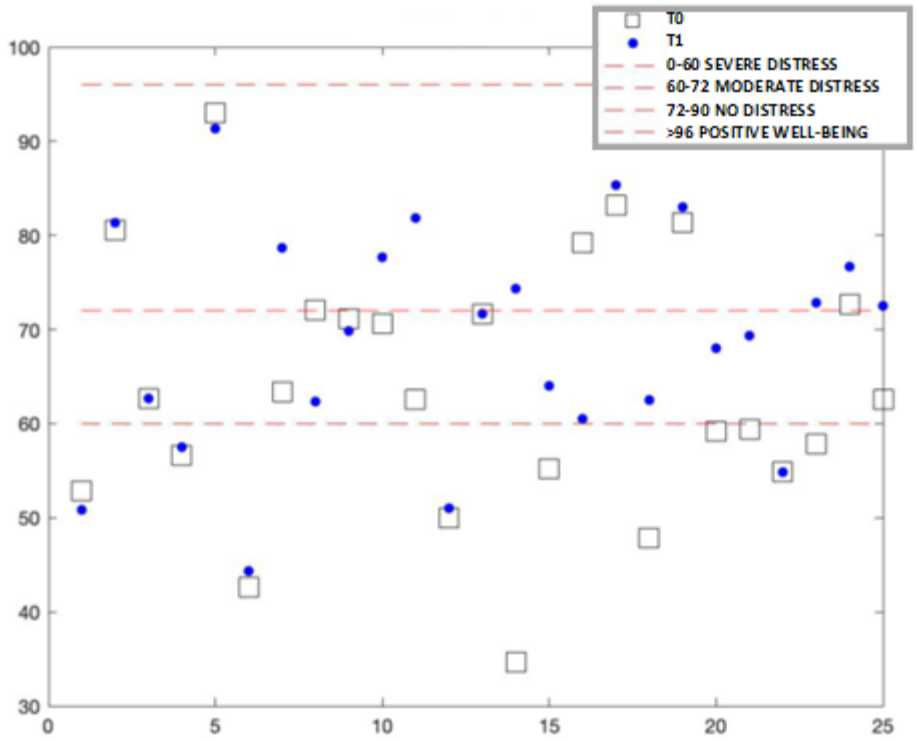
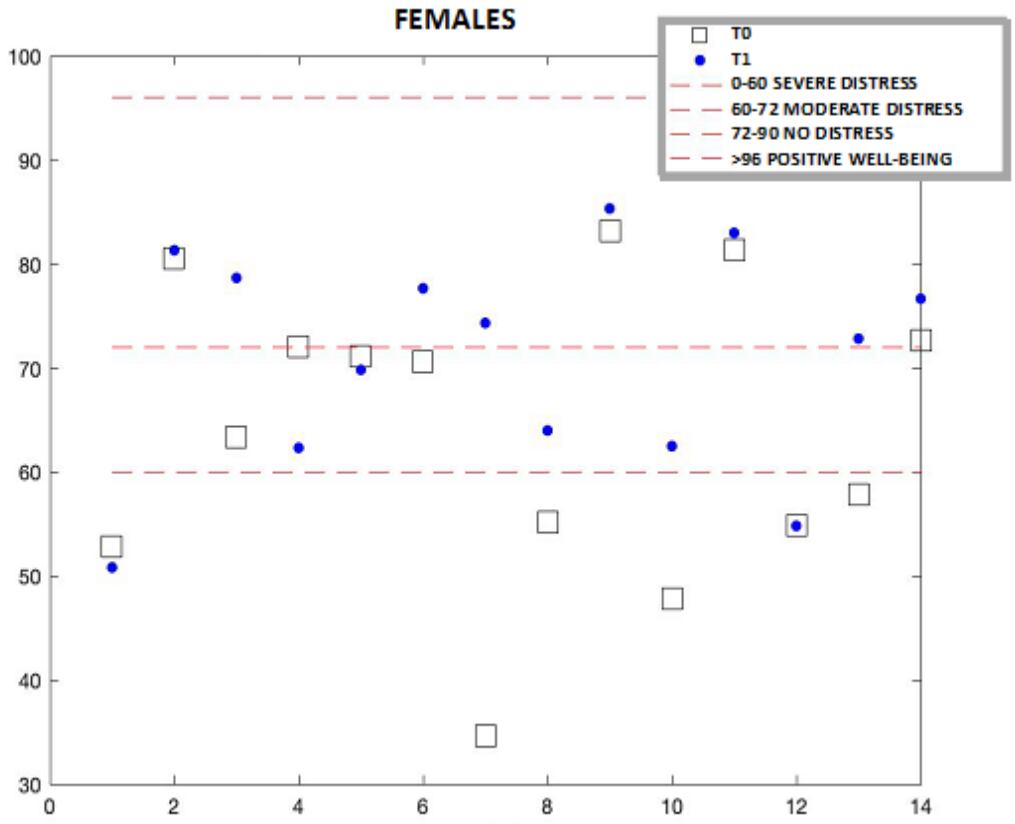
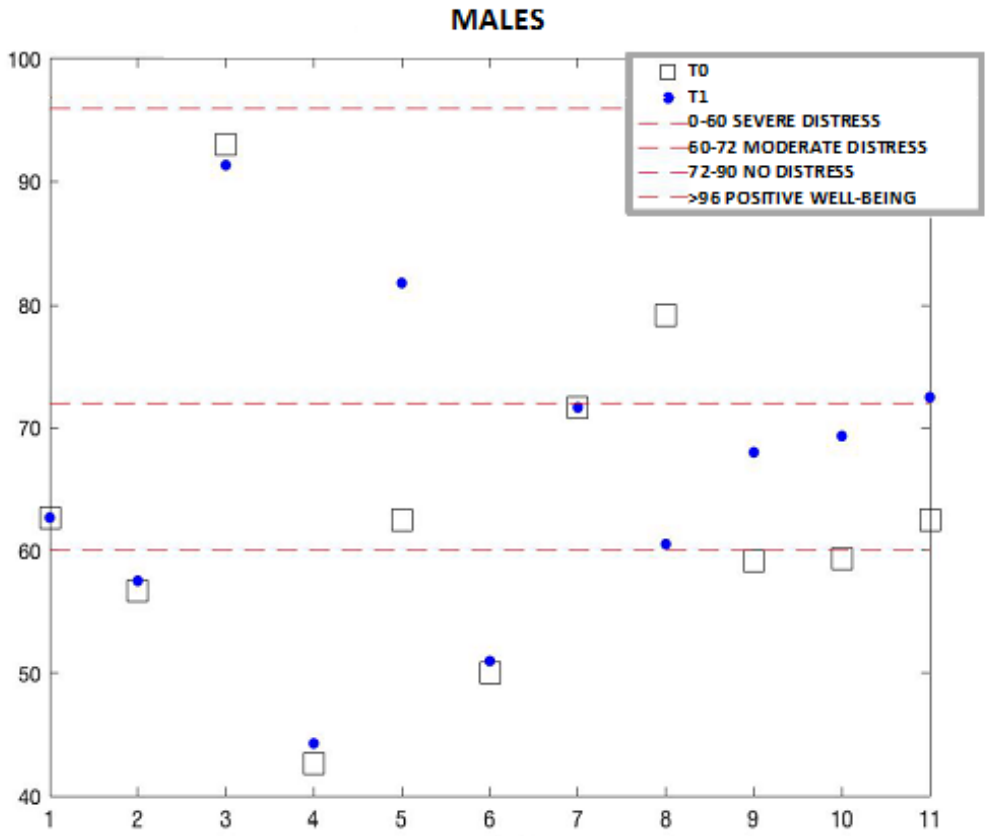
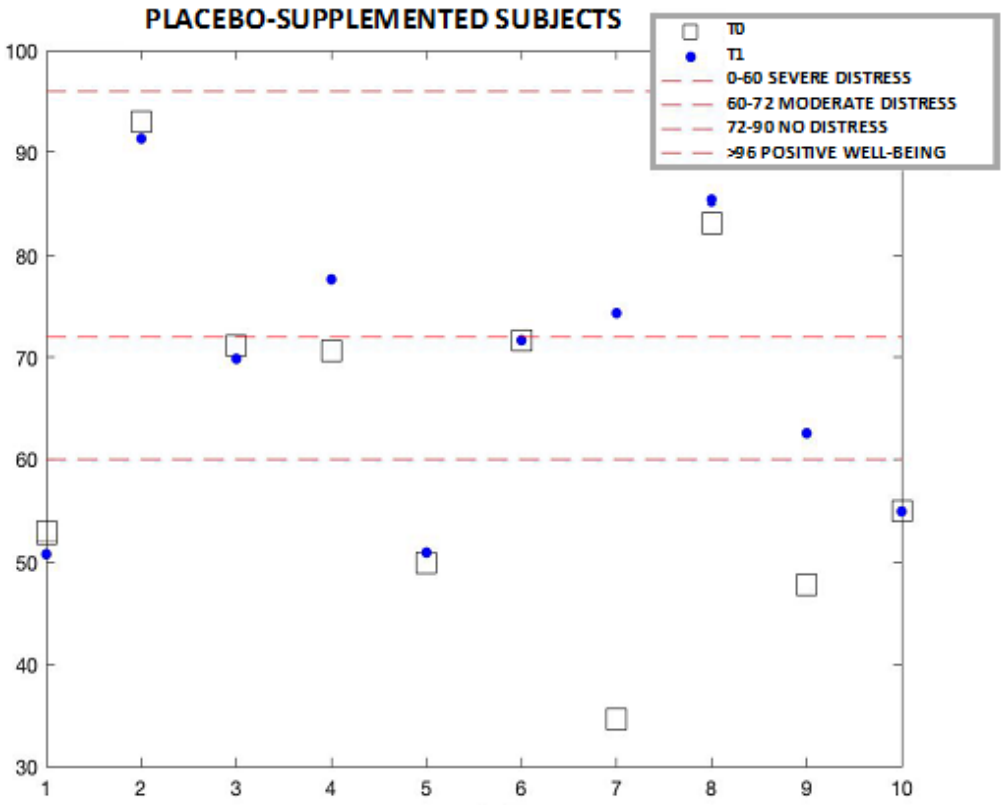


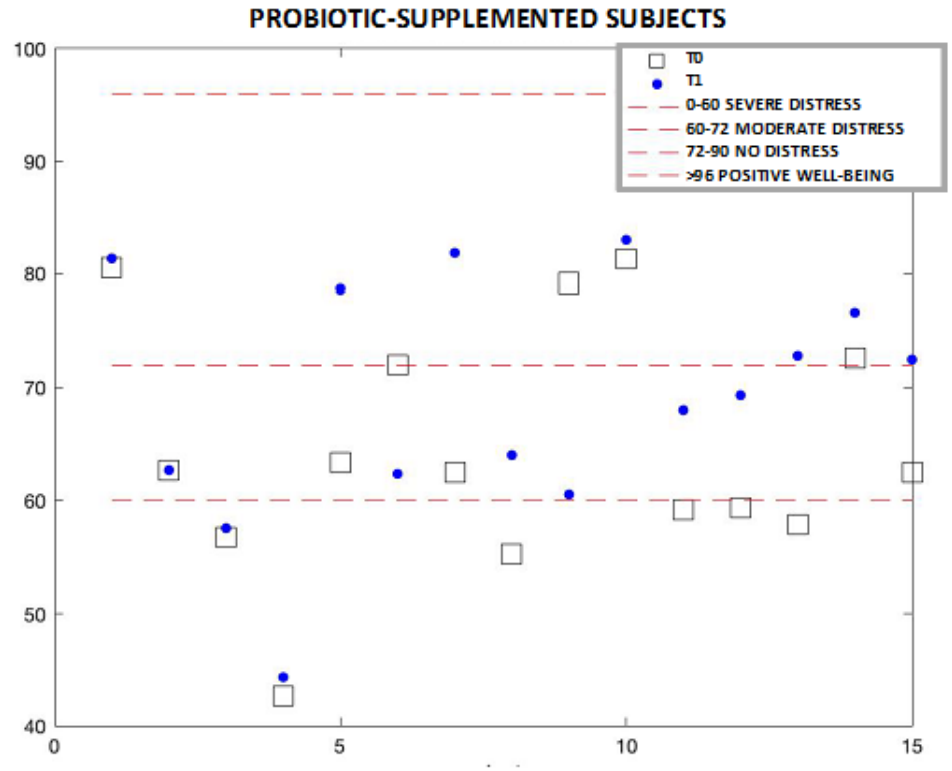
Figure 9. Comparison of PGWBI scores at T0 and T1 of all subjects.





Figures 10 and 11. Comparison of PGWBI scores at T0 and T1 of the probiotic supplemented group, basing on the time gender.





Figures 12 and 13. Comparison of PGWBI scores at T0 and T1 of all subjects, basing on the type of supplementation.

3.4.2.5 Bristol Stool Chart

The questionnaire submitted to volunteers and boarding home operators with the purpose to highlight changes in functionality intestinal was not accepted. No observations could be made or conclude relevant assessments, because the data were considered insufficient: only three subjects completed the questionnaire at both T0 and T1.

3.5 DISCUSSION AND CONCLUSIONS

Through the questionnaires survey, we obtained information on several aspects of life and health of the elderly population under study. Our results, collected before and after the dietary intervention, allowed to understand whether the supplementation contributed to improve the healthy ageing and the general well-being.

The PGWBI showed a considerable effect of the probiotic supplementation, suggesting it may be a responsive instrument to measure the impact of interventions on well-being and quality of life of elderly people. At baseline, the PGWBI described the population in a condition of moderate distress. The Index, calculated as mean score, increased at T1 regardless the type of supplementation, describing a general improvement in 68% of cases. The gender did not influence this variation, since no significant differences were recorded between females and males. Although this improvement was referred to both groups, the rate was higher after probiotic intervention (80% of subjects) than after the placebo one (50% of subjects). This interesting finding was supported by the analysis of the six individual domains of the questionnaire that confirmed the changes basing on the type of treatment administered.

Regarding the nutritional profile, assessed through the MNA questionnaire, both probiotic and placebo supplemented groups showed promising variations respect to baseline profile. Although most people were in the range “at risk of malnutrition” before and after the intervention, our study showed an evident impairment in nutritional status in placebo (61.5% of cases) than probiotic group (37.5%). Moreover, our results revealed that a longer supplementation is more effective than a shorter one. These findings suggest a potential influence of probiotic functional foods on nutritional status of elderly, but it needs further investigation.

On the contrary, the lifestyle and eating habits, as well for pathological status and pharmacological therapy, did not show relevant changes during the six months of the study.

In conclusion, despite the limitations of the human trial, such as the small sample size, and the complex nature of gut-brain interactions, our results suggest the apparent benefits of probiotic supplementation on psychological well-being. Some studies supported the positive effects of probiotics on mental health and mood, although the modulation mechanisms and metagenomic influences of GUT microbiota are still relatively unknown (Pirbaglou et al., 2016). In our study, also the nutritional status of elderly was partially beneficially affected by the probiotic supplementation, highlighting its potential role in preventing malnutrition in elderly, as supported by literature (Hamilton-miller). However, further work is necessary to confirm these preliminary findings and determine long term outcomes.

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CHAPTER IV

ASSESSMENT OF PROBIOTICS EFFECTS ON GUT MICROBIOTA MODULATION IN THE ELDERLY

4.1 ABSTRACT

Ageing is characterized by a low-grade chronic inflammation that involves several body systems and is marked by elevated circulating levels of inflammatory mediators. This situation, called inflammageing, represents a risk factor for morbidity and mortality in elderly people. With ageing, also the gut microbiota is strongly influenced, and its composition could be altered, leading to dysbiosis, a condition related with the pathogenesis of many inflammatory diseases and infections. Moreover, intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens, especially in elderly. The modulation of diet and eating habits with the daily consumption of new probiotic functional foods could be a good strategy to shape the gut microbiota across the lifetime. In this double-blind placebo-controlled study, we investigated the gut microbiota composition of 97 elderly before (T0) and after a probiotic foods-based intervention (T1). At baseline, the Real-Time PCR analysis revealed an intestinal microbiota typical for healthy elderly people, dominated largely by the phyla Firmicutes and Bacteroidetes, followed by Actinobacteria and Proteobacteria. Taking into account the gender, differences between males and females have been observed for *Cl. coccoides-Eu. rectale* group only. At T1, statistically significant increase was observed for *Lactobacillus* spp. in probiotic group respect to placebo one; moreover, the effectiveness of the intervention resulted in improved after 6-month duration than less. Other significant changes were referred to *Staphylococcus* spp. and *Bifidobacterium* spp., which decreased respectively after probiotic and placebo supplementation. The 16S NGS analysis on probiotic group showed a decreasing trend of Proteobacteria at T1 and conversely, an increasing trend of Actinobacteria and Verrucomicrobia phyla, to which the increase of Akkermansiaceae and Bifidobacteriaceae contributes at the family level. Overall, this study demonstrated that the probiotic-based

intervention had effects on the modulation of gut microbiota, suggesting it as an effective strategy to prevent dysbiosis, age-related morbidities and improve well-being in elderly.

4.2 INTRODUCTION

The human gastrointestinal (GI) tract represents one of the largest interfaces (250–400 m^2) between host, environmental factors and antigens in the human body (Bengmark, 1998). Up to now, the ecosystem of the GI tract has been the most intensively studied body environment as it stores the largest and diverse microbial population (Lloyd-Price et al., 2016). The collection of bacteria, archaea and eukarya colonising the GI tract is termed the 'gut microbiota' and has co-evolved with the host over years to form a close and mutually beneficial relationship (Bäckhed et al., 2005; Neish, 2009). The number of microorganisms inhabiting the GI tract has been estimated to exceed 10^{14} (Bäckhed et al., 2005; Gill et al., 2006). The gut microbiota forms an additional acquired organ that provides many essential functions to the host (Kodukula et al., 2017). A high inter-individual variability characterizes the composition of the gut microbiota, evolving throughout an individual's lifetime and it is affected by both exogenous and endogenous changes. In each person, the density and composition of microorganisms change along the GI tract due to different environmental, chemical, and physical conditions. Along the gut axis, the number and the variety of microorganisms change. The lowest bacterial number is found in stomach and small intestine, in which pH is low and the transit time is short, allowing to survive just a limited number of bacteria. On the other hand, the highest biodiversity is in the colon, where cell turnover rate and redox potential are low, and the transit time is longer, selecting the most suitable microbes for each environment (Thursby and Juge, 2017). The colonization of human gut begins during and after birth, but the composition of gut microbiota changes continuously (Kodukula et al., 2017; Nagpal and Yamashiro, 2018; Ottman et al., 2012).

The composition of intestinal microbiota is influenced by factors as geography, diet-related interactions, and age-related modifications (Mueller et al., 2006; Nagpal and Yamashiro, 2018). In the early stages of life, the microbiota has low diversity and it is dominated by two main phyla, Actinobacteria and Proteobacteria. Subsequently, the diversity increases, and the microbiota composition evolves towards an adult-like configuration (Kodukula et al., 2017; Nagpal and Yamashiro, 2018). The adult age shows the highest diversity and complexity, a condition that remains relatively stable during most of a healthy adult's life (Ottman et al., 2012). At this age, approximately 80% of the

total gut microbiota is integrated by members of the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria phyla (Claesson et al., 2011; Lloyd-Price et al., 2016). On the contrary, since the large inter-individual differences, it is hard to define a "typical" intestinal microbiota of elderly people (Biagi et al., 2012). Object of several studies, elderly gut microbiota seems to be characterized by a reduced bacterial diversity, changes in the dominant species and a consequent decrease in the availability of total short chain fatty acids (SCFAs). Comparing the microbiota of elderly with that of younger adults, lower levels of Firmicutes, mainly *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii*, and Actinobacteria (mainly bifidobacteria), and increased populations of Proteobacteria have been found. Regarding other relevant populations, such as Bacteroidetes phylum, the results are more variable, with some studies reporting lower levels, while others indicate increases of this microbial group in elderly subjects. Similarly, variable results have also been observed for lactobacilli (Salazar et al., 2017). Other studies found an unexpected similarity between the faecal microbiota of the young adults (aged 25–40 years) and elderly adults (aged 63–76 years), with Bacteroidetes and Firmicutes highly dominant (approximately 95%), a smaller fraction of Actinobacteria and Proteobacteria and comparable diversity values. In contrast, only centenaries >100 years old showed a significantly compromised gut microbiota. Bacteroidetes and Firmicutes are still dominant, but the relative proportion of Firmicutes subgroups changes. They also observed a significant decrease of bifidobacteria, increased levels of *Akkermansia muciniphila* and Proteobacteria (Biagi et al., 2012). To better understand the relationship between the gut microbiota and a long-living host, Biagi *et al.* (2016) provided a further phylogenetic microbiota analysis in person >105 years old, compared to adults, elderly, and centenarians. They confirmed the presence of a core microbiota of highly occurring, symbiotic bacterial taxa with an abundance decrease along with ageing and an age-dependent increasing contribution of subdominant species.

The just mentioned shifts in the gut microbiota is a gradual process that occurs with ageing. Ageing is associated with a time-dependent decline in physiological functions and capacity, with the accumulation of molecular and cellular damages and changes (Biagi et al., 2010; O'Toole and Jeffery, 2015). The age-related changes are mainly referred to GI tract, lifestyle and dietary schedule, living situation, comorbidities, socio-demographic aspect (An et al., 2018). For all these reasons, ageing can seriously affect human gut

microbiota composition and, consequently, human health and diseases development. The gut microbial community is widely recognized as a key component of GI homeostasis. It has protective, structural and metabolic functions, including helping to degrade food particles, breaking down xenobiotics and carcinogens, producing anti-inflammatory factors, SCFAs and essential vitamins, and providing protection against bacterial pathogens. The different bacterial groups can also stimulate the development of the mucosal and humoral immune system and the regulation of the intestine (Linares et al., 2017). On the contrary, loss of beneficial microbial organisms and diversity, with the increase of potentially harmful microorganisms, are events that encompass an altered gut status, called dysbiosis (Petersen and Round, 2014). Age-related changes in the intestinal microbiota, associated with increased systematic chronic inflammation, have been proposed to be responsible for the higher susceptibility of elderly people to recurrent and persistent infections and diseases (Salazar et al., 2017; Nagpal and Yamashiro, 2018).

The study of microbial composition has allowed to investigate the possible connections between microbiota and the most typical pathologies of the elderly people. Moreover, given that the intestinal microbiota is involved in the regulation of multiple metabolic pathways of the host giving rise to a series of interactive metabolic “axes”, with ageing it has been observed an altered communication among these axes. One of the most problematic expressions of population ageing is frailty, a multidimensional syndrome characterized by the reduction of functional reserves, resulting from the decline of multiple physiological systems that increases the risk of falls, hospitalizations, worsening disability, and mortality (Cesari et al., 2014). Convincing evidence suggests a relationship between microbiota alteration and fragility in the elderly. In 2016, a study conducted on 728 twin women with different degrees of fragility described the most and less abundant bacterial populations in frail individuals (Jackson et al., 2016). Another study showed that individuals with the high frailty score had a significant reduction in lactobacilli species when compared to non-frail individuals. This group also showed a decrease of bacteria within the *Bacteroides/Prevotella* and *Faecalibacterium prausnitzii* groups (van Tongeren et al., 2005). Sarcopenia, characterized by a progressively decline of the skeletal muscle mass and function, seems too, having a link with the intestinal microbial ecosystem (Shlisky et al., 2017). The role of gut microbiota in skeletal muscle regulation may occur through endocrine pathway, beginning with a disturbance in gut

homeostasis and culminating with alterations in skeletal muscles. It has been supposed that the reduced integrity of epithelial tight junctions, and the consequent increased intestinal permeability, facilitates the passage of endotoxin and other microbial products into the blood circulation. Once in the blood, they promote systemic inflammation that appears to trigger maladaptation of skeletal muscle (Grosicki et al., 2018). Moreover, a link between intestine and brain has long been hypothesized (Di Sabatino et al., 2018). Growing evidence suggests that gut microbiota is a key player in bidirectional communication across the gut–brain axis, even if the exact mechanisms underlying the communication need to be further elucidated. The routes of communication include the vagus nerve, gut hormone signalling, the immune system, tryptophan metabolism or by way of microbial metabolites such as SCFAs (Dinan and Cryan, 2017). The role of the microbiota on the regulation of this axis is critical for physiological processes, and an altered microbiota has been implicated in a variety of neurodegenerative disorders, including depression, autism, schizophrenia, Alzheimer's disease and Parkinson's diseases (Sánchez-Garcí-a et al., 2017). Alzheimer's disease (AD) is the most common cause of dementia and has an important impact on the elderly population (Ballard et al., 2011). Although AD has a complex pathogenesis and its aetiology is not well understood, intestinal microbiota seems to play a role in this process. In particular, *in vivo* and *in vitro* studies have shown a correlation with the production of LPS, one of the most important products of the microbiota. Studies carried out on murine specimens have shown that LPS can promote the formation of amyloid bridles and their deposition, finding higher levels of LPS and *E. coli* fragments in Alzheimer's disease patients than the control (Zhan et al., 2016). Several recent experiments support that gaining the maximum health benefits from the microbiota may require a more complex and diverse collection of microorganisms (Petersen and Round, 2014). As explained before, the ageing process promotes changes (lifestyle and dietary habits) in elderly people that contribute to the reduction in the abundance and diversity of the intestinal microbiota, so this goal may be even more difficult to achieve. This condition, in turn, promotes also inflammageing and, consequently, the risk of all the age-related pathologies (Candela et al., 2014; Shanahan et al., 2017). For all these reasons, it is important to find strategies to improve and ensure the well-being of the intestine, since its key role for human health, especially in elderly people. In this study, we aimed to evaluate the effects of a probiotics-based

supplementation on the modulation of gut microbiota. We monitored the intestinal microbiota of volunteers at baseline conditions and after the intervention, comparing the obtained results with a placebo group, used as control.

4.3 MATERIALS AND METHODS

4.3.1 Faecal samples

The volunteers' faecal samples were frozen immediately after sampling by the nursing staff and then transferred to the laboratory, where they were processed for the enumeration of selected bacterial groups to have the general profile of composition of intestinal microbiota at baseline status and to monitor the changes at different time of supplementation. The first time point of the sampling represents the baseline status of the subjects. The faecal sampling and analysis were repeated after 3 or 6 month-dietary intervention.

4.3.2 DNA extraction

The stool DNA extraction was performed using the "Norgen's Stool DNA Isolation Kit" (Norgen Biotek Corporation, Thorold, Canada), following the manufacturer's instructions. It provides a convenient and rapid method to isolate total DNA from fresh or frozen stool samples.

4.3.3 Real-Time PCR analysis

The Real-Time PCR analysis was run to detect and quantify selected bacterial groups: *Bacteroides -Prevotella-Porphyromonas* spp., *Staphylococcus* spp., *Clostridium coccoides–Eubacterium rectale* group, *Lactobacillus* spp., *Bifidobacterium* spp., and Enterobacteriaceae. The reference strains used for the bacterial groups were: *Bacteroides fragilis* DSM 2151, *Staphylococcus aureus* ATCC 25923, *Blautia producta* DSM 2950, *Lactobacillus acidophilus* ATCC 314*, Bifidobacterium longum* DSM 20219, *Escherichia coli* ATCC 13706 and the respective specific primers, as reported in Table 1 (Nasuti et al., 2016).

Table 1. List of bacterial groups, respective reference strains and forward and reverse primer sequences.

TARGET	PRIMER SEQUENCE (5'-3')	REFERENCE STRAIN
Bacteroides-Prevotella-Porphyromonas spp.	Fw: GGTGTCGGCTTAAGTGCCAT Rv: CGGAYGTAAGGGCCGTGC	*Bacteroides fragilis* DSM 2151
Staphylococcus spp.	Fw: GCGATTGATGGTGATACGGTT Rv: AGCCAAGCCTTGACGAACTAAAGC	*Staphylococcus aureus* ATCC 25923
Clostridium coccoides-Eubacterium rectale group	Fw: CGGTACCTGACTAAGAAGC Rv: AGTTTYATTCTTGCGAACG	*Blautia producta* DSM 2950
Lactobacillus spp.	Fw: TGGAAACAGRTGCTAATACCG Rv: GTCCATTGTGGAAGATTCCC	*Lactobacillus acidophilus* ATCC 314
Bifidobacterium spp.	Fw: GGGTGGTAATGCCGGATG Rv: TAAGCGATGGACTTTCACACC	*Bifidobacterium longum* DSM 20219
Enterobacteriaceae	Fw: CATTGACGTTACCCGCAGAAGAAGC Rv: CTCTACGAGACTCAAGCTTGC	*Escherichia coli* ATCC 13706

SYBR Green Real-Time PCR amplification was performed using an iCycler iQ Real-Time Detection System (Stratagene, La Jolla, California) associated with MXP Software and following a specific protocol. The conditions and the standard curves used for each bacterial group are presented in table 2 (Avella et al., 2010; Nasuti et al., 2016).

Table 2. Real-Time PCR conditions run of each group of bacteria.

REFERENCE STRAINS	DENATURATION	ANNEALING TEMP.	EXTENSION	CYCLES	PRODUCT SIZE (bp)
Bacteroides fragilis DSM 2151	95°C 15 s	58°C 20 s	72°C 30 s	35	140
Staphylococcus aureus ATCC 25923	95°C 15 s	49°C 20 s	72°C 30s	35	279
Blautia producta DSM 2950	95°C 15 s	55°C 20 s	72°C 30 s	35	429
Lactobacillus acidophilus ATCC 314	95°C 15 s	47°C 1 min	72°C 1 min	40	230
Bifidobacterium longum DSM 20219	95°C 30 s	59°C 30 s	72°C 45 s	35	457
Escherichia coli ATCC 13706	95°C 30 s	55°C 30 s	72°C 45 s	35	195

4.3.4 NGS analysis

The NGS analysis was performed with the support of SYNBIOTEC Srl (Camerino, Italy), that was involved in PROBIOSENIOR project as coordinator.

From the volunteers' faecal samples, collected into sterile tubes and stored in a −80°C cryogenic freezer, the microbial DNA extraction was performed (as specified before) and DNA was also used for the next generation DNA sequencing. The V3-V4 hypervariable regions of 16S rDNA were amplified using universal primers (341F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', 805R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3') following the 16S Metagenomics Sequencing Library preparation protocol (Part #

15044223 B). Libraries were sequenced using the MiSeq Illumina Platform (Illumina Inc. San Diego, CA, USA) with a 2x250 paired end run. Poor quality reads were filtered with Trimmomatic (Bolger et al., 2014); paired-ends reads were merged using FLASH (Magoč and Salzberg, 2011) and processed with VSEARCH (Rognes et al., 2016) to detect potential chimera sequences and to cluster merged amplicons in operational taxonomic units (OTUs), with a minimum pair-wise identity threshold of 97%. The NCBI 16S RefSeq database (O'Leary et al., 2016) was employed for taxonomic classification. Evaluation of microbial alpha (Adv, Chao1, Simpson's and Shannon's diversity) and beta (UniFrac distances, Bray–Curtis dissimilarity) diversity measures were performed using an internal pipeline of SYNBIOTEC Srl. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways potentially affected by groups of bacteria.

4.3.5 Statistical analysis

All assays were performed in duplicate, the results were expressed as mean ± standard deviation. Paired, unpaired Student's t test and Tukey's Multiple Comparison Test were used to detect differences among mean values of results obtained at different time points, basing on different variables. $P < 0.05$ was regarded as significant.

4.4 RESULTS

4.4.1 Gut microbiota composition at baseline

4.4.1.1 Real-Time PCR analysis

At baseline conditions, intestinal microbiota of the 97 enrolled volunteers was characterized at genus level, through qPCR analysis. The subjects randomly allocated into the probiotic group were 59 and the subjects into the placebo were 38.

Figure 1 shows the faecal bacterial cell counts of the six intestinal bacterial groups of interest: *Bacteroides-Prevotella-Porphyromonas* spp., *Staphylococcus* spp., *Cl. coccoides-Eu. rectale* group, *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterobacteriaceae*. The intestinal microbial environment observed before the intervention (T0) was typical for healthy individuals, largely dominated by the phyla Firmicutes and Bacteroidetes, followed in smaller proportion by Actinobacteria and Proteobacteria (Eckburg et al.,

2005). In details, *Bacteroides-Prevotella-Porphyromonas* spp. mean cell count was 8.27 Log CFU/g of faeces; *Staphylococcus* spp. and *Bifidobacterium* spp. presented low variability within the group analysed. *Staphylococcus* spp. showed mean value of 6.8 Log CFU/g of faeces, while *Bifidobacterium* spp. cell count was 7.7 Log CFU/g. *Cl. coccoides-Eu. rectale* group presented mean value of 8.04 Log CFU/g of faeces. *Lactobacillus* spp. and Enterobacteriaceae cell counts were similar, with mean values respective of 6.79 and 6.68 Log CFU/g of faeces.

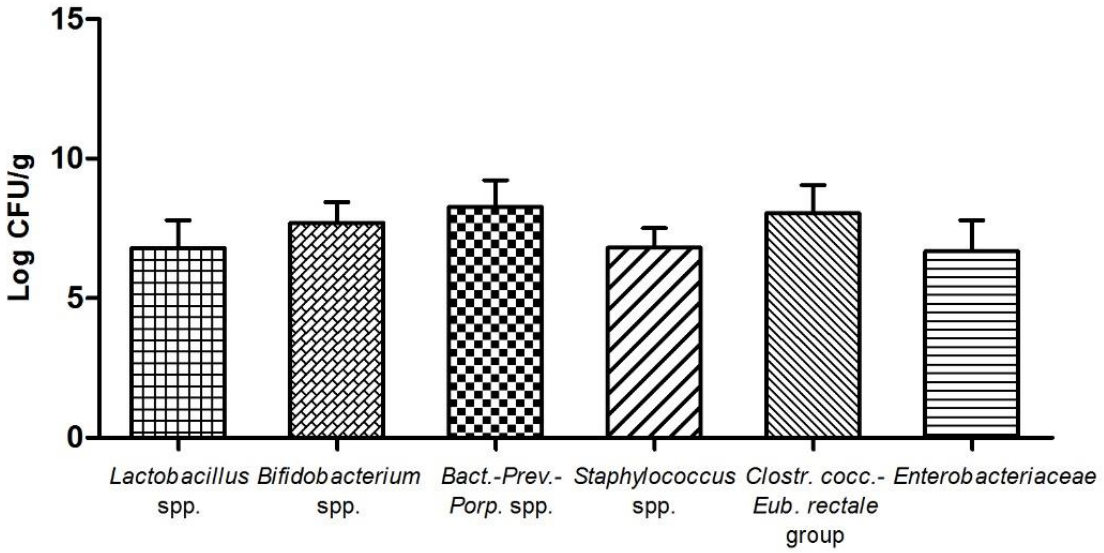

Figure 1. Faecal bacterial cell counts of target bacterial groups, at baseline, relative to the total enrolled volunteers. The values are expressed as Log CFU/g of faeces (mean ± SD; n=97).

The results obtained were further elaborated considering the gender and the residence structure of subjects, to have a more complete overview on the senior population characteristics at baseline.

Figure 2 A shows the bacterial counts classifying all subjects according to gender. The differences between genders could be affected by the hormonal profile and balance, and some intestinal bacterial groups or phyla are stronger influenced than others, as explained in several studies (Shin et al., 2019). Three bacterial groups out of six (*Bifidobacterium* spp., *Staphylococcus* spp., Enterobacteriaceae) had very similar mean values between the female and male groups, with no significant differences. Gender differences were observed for *Bacteroides-Prevotella-Porphyromonas* spp. with higher levels in males than females, as confirmed in some studies (Mueller et al., 2006; Koliada

et al., 2021). A similar trend was shown in *Cl. coccoides-Eu. rectale* group, with statistically significant differences between male and female groups. For *Lactobacillus* spp., cell count in female group was higher, although not significant, than in male group. Other differences among genders observed at phylum-level are related to the Firmicutes-Bacteroidetes (F/B) ratio, that was higher in females compared to males. These results were also supported by a study conducted on the relative abundance of major gut microbiota phyla in male and female participants (Koliada et al., 2021).

Figure 2 B also shows the arrangement of data basing on the residence structure of subjects. It is interesting to understand how the profiles differ in people living in boarding homes and in private houses, since the microbiota profile is strongly influenced by the diet but also by the combination of different environmental factors, as reported by several studies (Deschasaux et al., 2018; Mueller et al., 2006). The baseline levels of *Staphylococcus* spp., *Cl. coccoides-Eu. rectale* group, Enterobacteriaceae and *Bacteroides-Prevotella-Porphyromonas* spp. presented slight but not statistically significant differences between free-living and boarding home subjects. *Bifidobacterium* spp. cell count seems to be not affected by the different diet and environment, since the two groups didn't show variations. On the contrary, the average level of lactobacilli at baseline was statistically higher in free-living than in boarding homes subjects.

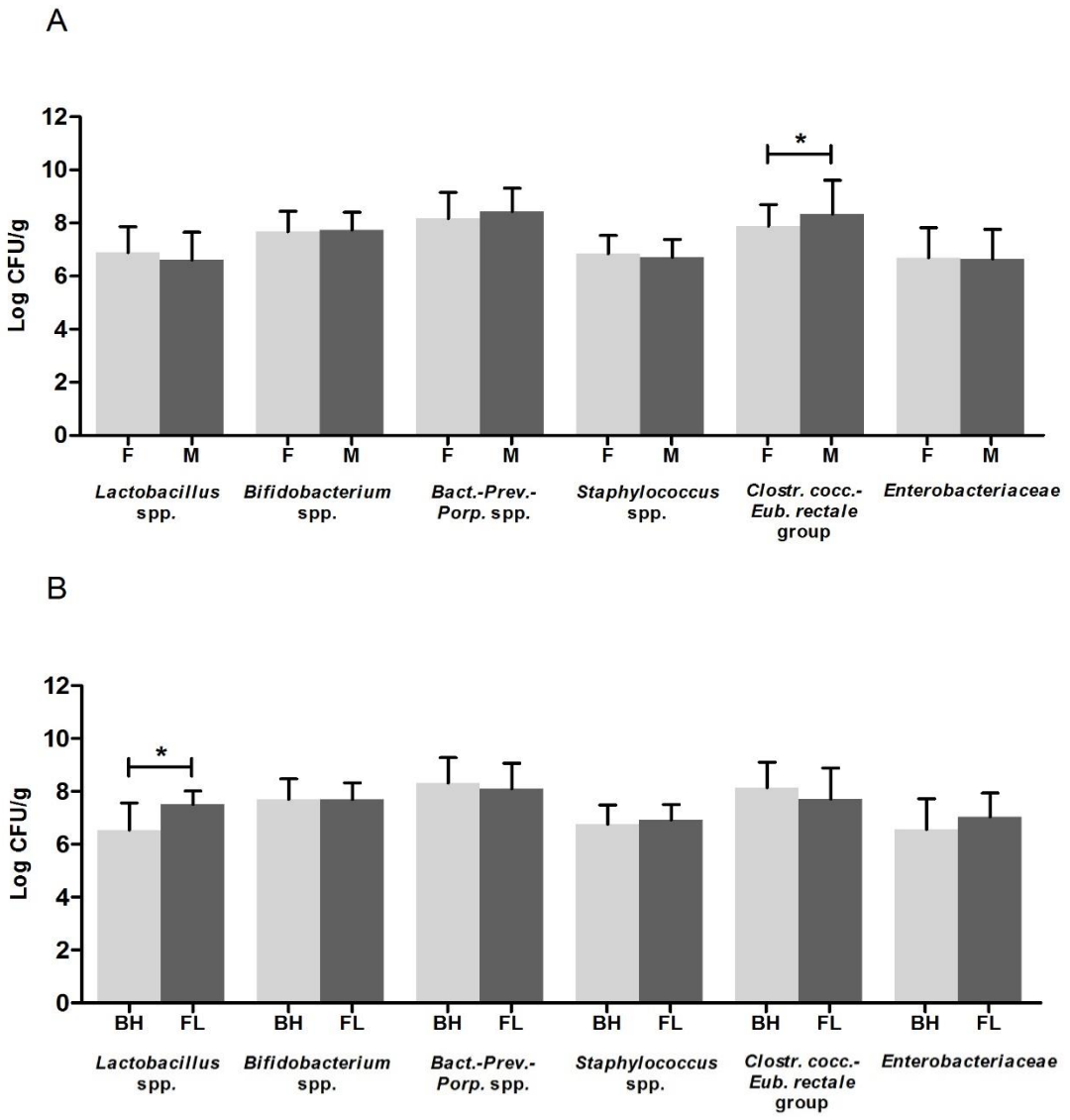

Figure 2. Faecal bacterial cell counts of target bacterial groups, detected in faecal samples at baseline, according to gender (A) and residence structures (B) of the senior population. F= female; M= male. BH: boarding home group; FL: free-living group. Values expressed as Log CFU/g of faeces (mean ± SD; n=97); *Statistically significant difference by unpaired Student's t test (P<0.05).

4.4.2 Gut microbiota composition after intervention

4.4.2.1 Real-Time PCR analysis

The intestinal microbiota profile was studied before and after the intervention, and the first data analysis was carried out considering the type of supplementation as factor influencing the composition of the gut microbiota.

Figure 3 shows the results of bacterial counts at genus level obtained after both supplementations. In placebo group, some slight variations were observed in most

bacterial groups, with no statistical significance. Only in *Bifidobacterium* spp., a significant decrease was recorded at time T1. On the contrary, after probiotic intervention, *Bifidobacterium* spp. cell count increased, although not in significant manner. However, it is interesting to analyse the proportion of subjects with increased/decreased bifidobacterial concentration after both interventions, because it tended to be different (Chi square, p=0.001). In particular, bifidobacteria decreased in 24 out of 38 subjects (63%) in placebo group, while an opposite trend was observed in probiotic group, with an increase in concentration in 45 out of 59 (76%) participants.

For the other bacterial groups, the main change after probiotic intervention was presented in *Lactobacillus* spp., with a significant increase at time T1 respect to T0 and placebo group. *Bacteroides-Prevotella-Porphyromonas* spp. doesn't seem to be affected by both supplementations, maintaining stable concentrations at T1. A common but not significant trend was observed in probiotic and placebo groups for *Staphylococcus* spp., *Cl. coccoides-Eu. rectale* group and Enterobacteriaceae levels. In details, both interventions seem to decrease *Cl. coccoides-Eu. rectale* group and *Staphylococcus* spp. cell counts, while Enterobacteriaceae concentration, physiologically higher in elderly respect to young adults[42], increased at T1 (Figure 3).

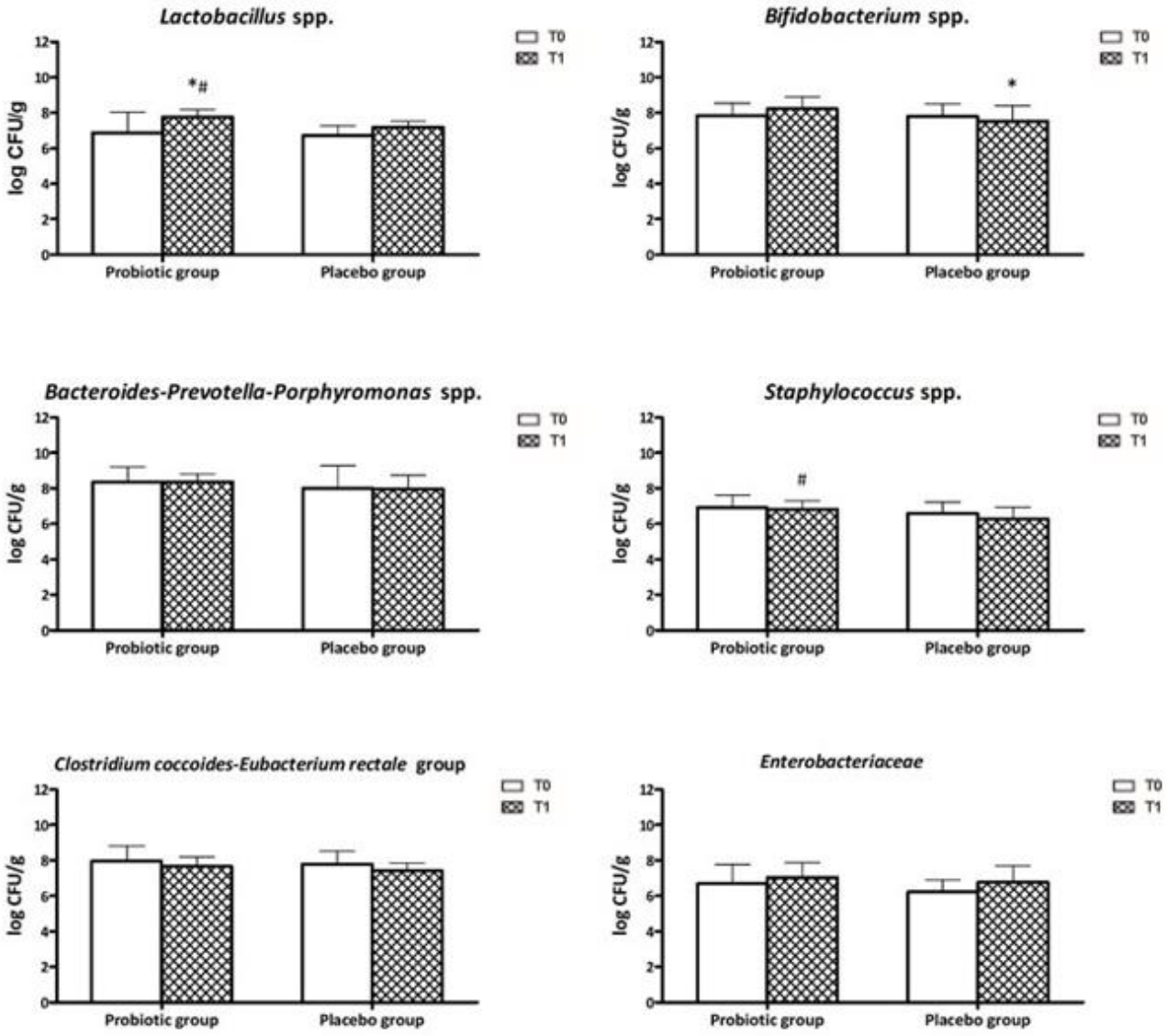

Figure 3. Faecal bacterial cell counts of target bacterial groups at two different time points (T0, T1) in the two groups of subjects (Probiotic and Placebo group). Values expressed as Log CFU/g of faeces (mean ± SD; n=97); *Significantly different ($P<0.05$) from T0 and #from Placebo group by Tukey's test, One-way ANOVA.

Within the probiotic group, the time of supplementation seems to influence the effects on gut microbiota. Of the subjects enrolled and allocated in the probiotic group, 33 out of 103 didn't complete the experimental phase. For this limited number of subjects, the microbial profile was studied after 3 months of probiotic supplementation instead of 6. These incomplete analysis data allowed the evaluation of the effects of short and long-term probiotic supplementation on gut microbiota. The main findings were referred to *Lactobacillus* spp., where the 6 months supplementation positively influenced its effectiveness, leading to a significant increase in cell count in 100% of subjects. On the

contrary, the values remained unchanged after a shorter supplementation period (Figure 4 A). Overall, no significant variations in the faecal abundances of all other bacterial groups were observed comparing the duration of probiotic intervention (Figure 4).

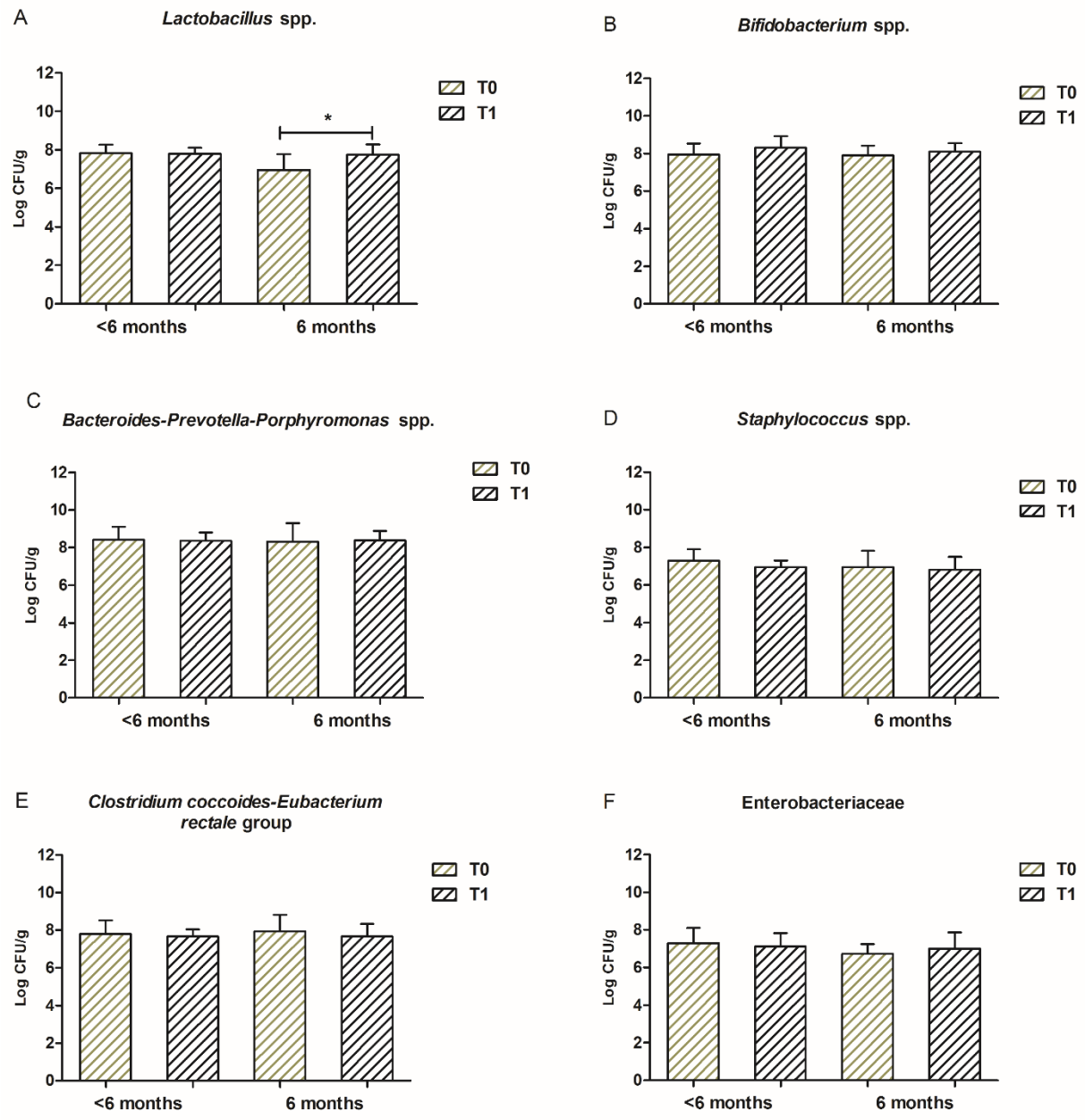

Figure 4. Faecal bacterial cell counts of target bacterial groups at two different time points (T0, T1) in the groups of subjects supplemented with probiotics for 6 months (n=59) or less than 6 months (n=33). Values expressed as Log CFU/g of faeces (mean ± SD; n=97); *Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.

The second data analysis was performed comparing the changes between before and after intervention, taking into account the residence structure (Figure 5). Similar trends were observed in boarding homes and free-living subjects, also considering the type of supplementation received. The most evident changes were observed in boarding homes group, probably also due to the different samples size. *Lactobacillus* spp. levels raised in boarding home subjects after both interventions, but the increase was statistically significant after probiotic supplementation (Figure 5 A) only. For the other bacterial groups, some slight differences were observed in the two groups at T1, although without statistically significant variations (Figure 5 B-F). The results described in this second data analysis confirmed the findings explained previously. The differences observed between the two residence structures are probably due to diet, lifestyle and environment-related conditions that are peculiar and may influence some further cross-interactions (Mueller et al., 2006).

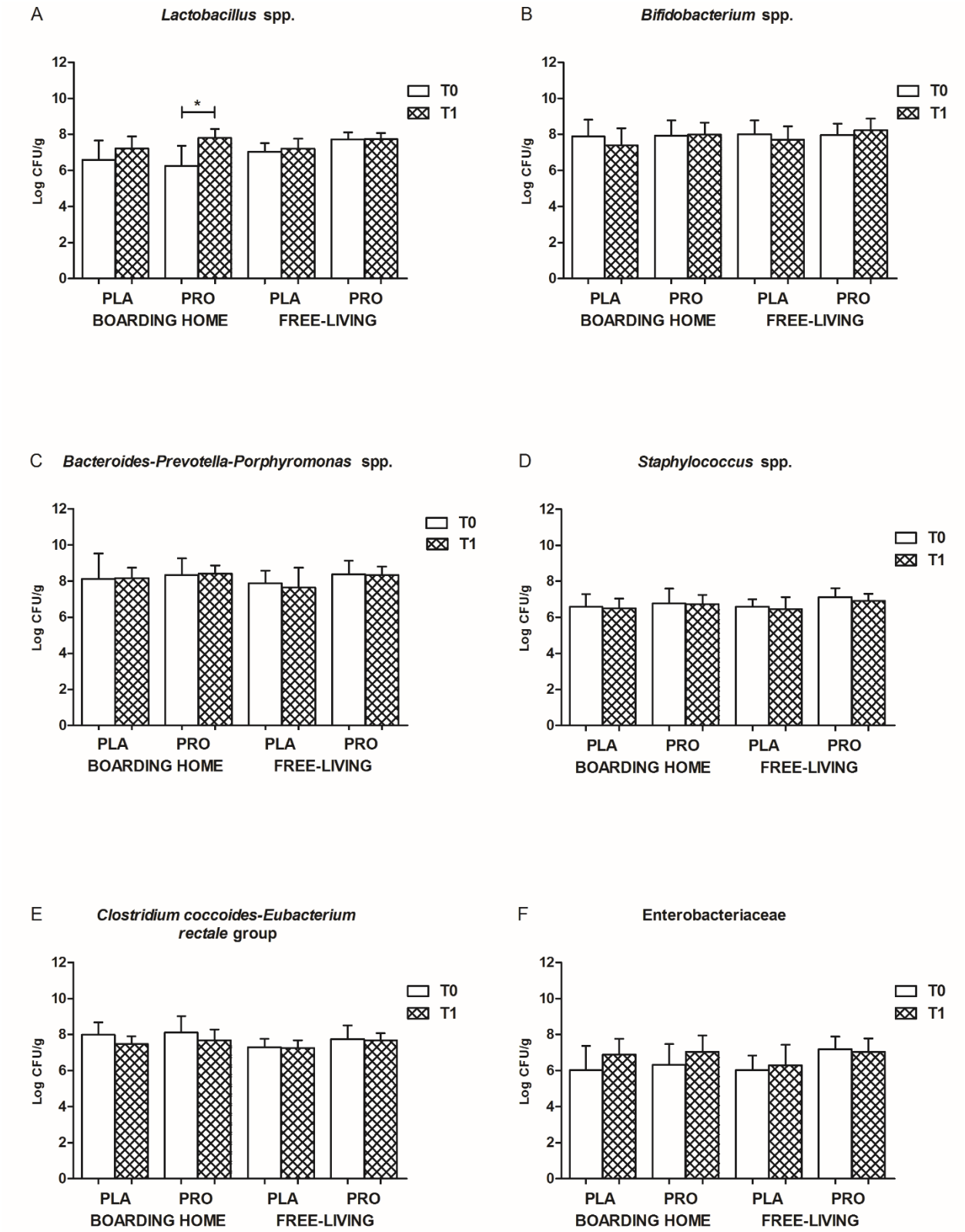

Figure 5. Faecal bacterial cell counts of target bacterial groups at two different time points (T0, T1) in the subjects enrolled in boarding homes and private houses. Values expressed as Log CFU/g of faeces (mean ± SD; n=97); *Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.

4.4.2.2 NGS analysis

V3-V4 regions of the 16S rRNA genes were sequenced using the Illumina MiSeq platform, to assess the effects of probiotic supplementation on the intestinal microbiota. We analysed the bacterial microbiota composition of all the samples in probiotic group at time T0 and T1. To evaluate the change of α-diversity before and after the probiotic supplementation, Shannon, Simpson, chao1 and adv indices were used.

The results are presented in Figure 6. In terms of species richness, no significant difference was observed ($p>0.05$). The Bray–Curtis distances used to reveal β-diversity, that is bacterial structural differences before and after the probiotic intervention, didn't show a congruent directional pattern between the two groups. At the phylum level, about 99% of the sequences belonged to the five most populated bacterial phyla in both time points, namely Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia and Actinobacteria (41.95% vs 40.68%, 44.17% vs 45.28%, 8.63% vs 5.72%, 1.84% vs 3.22% and 2.13% vs 3.93%, respectively) (Figure 7). At the family and genus level, 25 families and 27 genera were the dominants in both time points (relative abundance of >1%) (Figure 8 and 9). No significant difference in Firmicutes and Bacteroidetes abundance was identified after probiotic intervention (T1). Anyway, a significant change in the relative abundance of Proteobacteria, Verrucomicrobia and Actinobacteria was registered. Interestingly, Proteobacteria, which has been associated with increased gut inflammation and dysbiosis, was more abundant in seniors at T0 and it tends to decrease after the probiotic supplementation. On the other hand, the Actinobacteria and Verrucomicrobia phyla increased after probiotic intervention. Within the Verrucomicrobia phylum, the increase of the Akkermansiaceae and *Akkermansia* at the family and genus level respectively, is notable. Moreover, the levels of total bifidobacteria were positively affected by the probiotic supplementation as evidenced by significant increase in Verrucromicrobia at the phylum level, to which contribute an increase in Bifidobacteriaceae and *Bifidobacterium* at the family and genus level respectively. In addition, we explored the metabolic activity of the gut microbiota in probiotic group through PICRUSt analysis to predict the functional profiling of the microbial communities based on the 16S rRNA gene sequences. Interestingly, after the administration of probiotics, a significant increase in some metabolic pathways were observed. Probiotic use significantly modulated 8 functional

pathways, in particular some amine, aromatic compounds and amino acids degradation pathways.

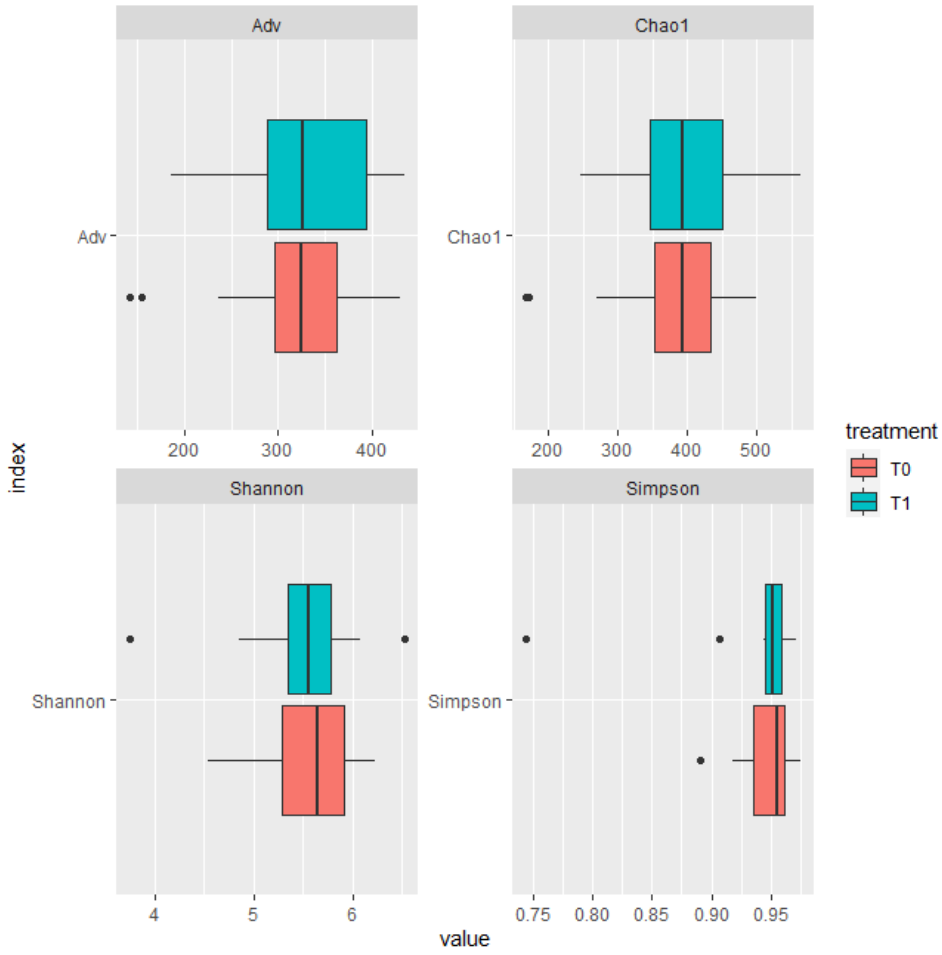

Figure 6. Comparison of alpha-diversity indices Adv, Chao1, Shannon and Simpson in faecal samples collected at T0 and T1 in subjects of probiotic group.

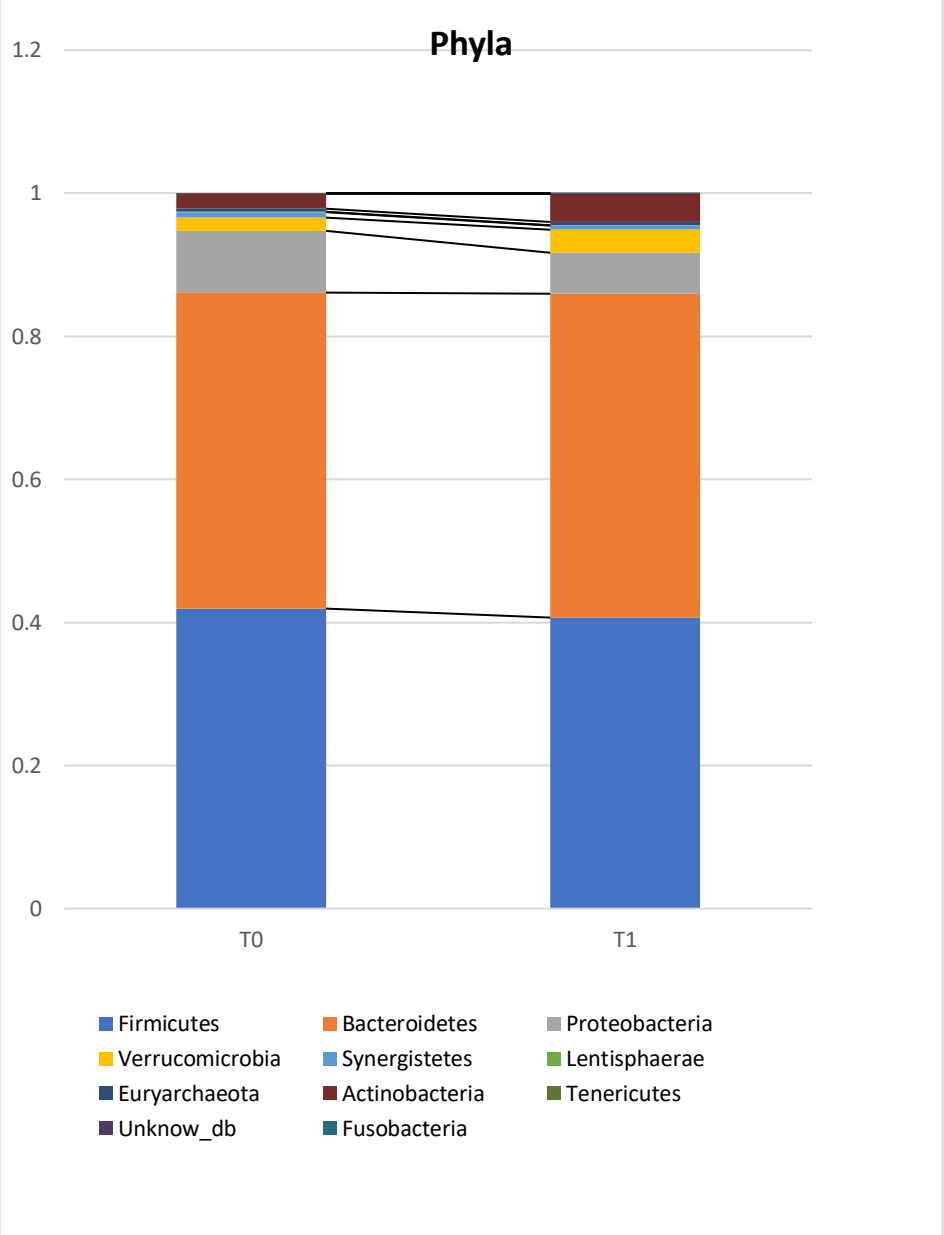

Figure 7. Relative abundance of the phyla. The x-axis represents sampling times (T0 and T1) and the y-axis represents relative abundance present.

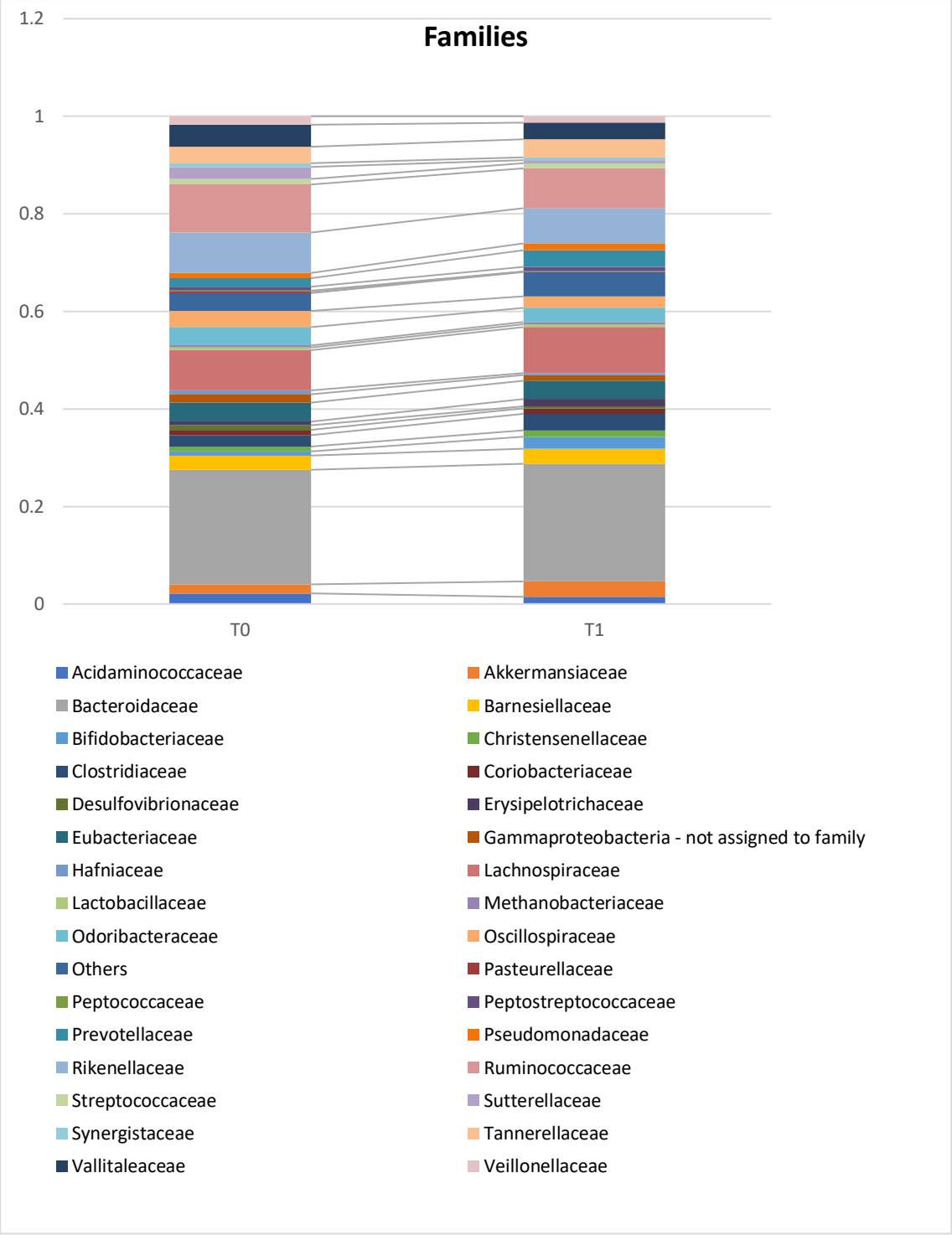

Figure 8. Relative abundance of the families. The x-axis represents sampling times (T0 and T1) and the y-axis represents relative abundance present.

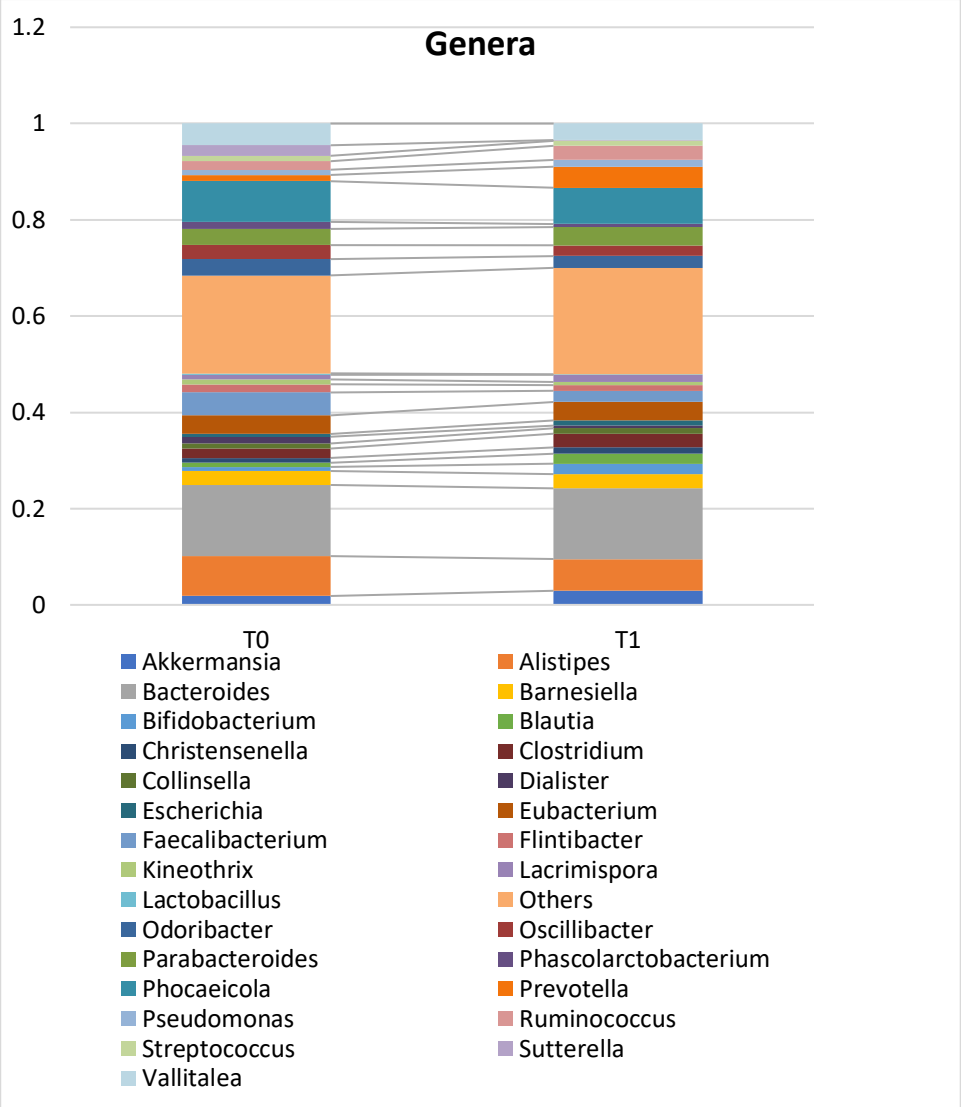

Figure 9. Relative abundance of the top 25 genera; other species were combined as "Others". The x-axis represents sampling times (T0 and T1) and the y-axis represents relative abundance present.

4.5 DISCUSSION AND CONCLUSIONS

The evaluation of the effects of the probiotic supplementation on intestinal microbiota composition of elderly volunteers was one of the main objectives of the study. Beside this, we also studied the microbial profile before the intervention, to have an overview of the baseline conditions of the community. The data were studied and elaborated focusing on all the variables that could influence elderly status, such as the gender, the diet, the living structures/conditions and the intervention time. As demonstrated in this study, gut microbiota is extremely variable between individuals and within each group analysed. In general, the data obtained at baseline confirmed that the

community under study had the typical gut microbiota composition of healthy elderly subjects, with Firmicutes and Bacteroidetes phyla are predominant, followed by Actinobacteria and Proteobacteria. Since the hormonal profile may influence the intestinal microbiota composition, the results were further organised considering the gender of the subject. Based on that, significant differences were observed for *Cl. coccoides-Eu. rectale* group with higher levels in males than females. Moreover, also at phylum-level intestinal microbial composition, the Firmicutes to Bacteroidetes ratio (F/B) revealed higher values in females compared to males. The other bacterial groups do not seem to be influenced by gender. The environmental conditions may also affect the gut microbiota composition in elderly; comparing the profiles of subjects living in boarding homes and private houses, the results revealed statistically higher lactobacilli cell count in free-living subjects than in boarding home group.

At bacterial genus level, significant differences were observed after probiotic intervention. With ageing, gut microbiota composition changes, with a reduction in *Lactobacilli* spp., *Bifidobacterium* spp., and *Bacteroides* spp. levels (Ragonnaud and Biragyn, 2021). On the contrary, in this study, *Lactobacillus* spp. cell count significantly increased after the probiotic supplementation. In addition, *Bifidobacterium* spp. increased in probiotic group after supplementation, although in a not significant manner; while in placebo group, the cell count decreased at the end of intervention.

The age-related modifications of intestinal microbiota also affect other bacterial groups, such as *Staphylococcus* spp., *Cl. coccoides-Eu. rectale* group, and Enterobacteriaceae. In this study, we observed a decrease in *Staphylococcus* spp. cell count in both groups, with a statistically significant difference just for probiotic group at T1 respect to T0. A positive trend was also observed for *Cl. coccoides-Eu. rectale* group, with a reduction in concentration after both interventions, despite its levels in elderly tend to increase due to the use of antibiotics and drugs. Moreover, our study revealed a common increased trend for Enterobacteriaceae after probiotic and placebo dietary intervention, but without significant changes. The changes occurred after the probiotic intervention, were further investigated comparing the composition of microbiota in relation of the residence structure of subjects. In most cases, we observed a big variability between the groups, with a significant increase at T1 respect to T0 in boarding homes subjects supplemented with probiotics. For the other bacterial groups, no significant

variations were observed considering the supplementation related with the residence structure.

Interesting is also the effect of the probiotic functional food-based diet considering the supplementation time. Some bacterial groups seem to be more influenced by a 6-month intervention, while others showed significant changes with a shorter supplementation. *Lactobacillus* spp. cell count significantly increased in 100% of subjects after 6 month-intervention, while no significant variation was observed after less than 6 month-duration. For the other bacterial groups, the intervention time doesn't seem to affect the effectiveness of the probiotic intervention with significant changes. Finally, results obtained by 16S NGS analysis revealed that probiotic product administration significantly affected some phyla, families, and genera abundance. After probiotic intervention, a significant increase of Bifidobacteriaceae family and *Bifidobacterium* genus was observed, highlighting the positive effects of probiotic supplementation on gut modulation.

In conclusion, the results obtained from this study are very interesting. As documented by literatures, in elderly community a high variability is observed and many factors may influence elderly conditions and, consequently, the subject's response to experimental intervention (Mueller et al., 2006). Despite this, in PROBIOSENIOR Project, probiotics-based intervention seems to exert a positive modulation on gut microbiota respect to the placebo control supplementation, especially after 6-month intervention. Since the strong connection between intestinal environment and human health, the obtained results support those probiotic functional foods could have a useful role in the improvement of elderly general well-being, preventing dysbiosis and age-related morbidities.

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CHAPTER V

PROBIOTICS EFFECTS ON SHORT-CHAIN FATTY ACIDS PRODUCTION BY GUT MICROBIOTA

5.1 ABSTRACT

Short chain fatty acids (SCFAs) are the main metabolites produced in the colon by bacterial fermentation of dietary fibres and resistant starch (Markowiak-Kopeć and Śliżewska, 2020). SCFAs play a key role in many human pathways, such as the regulation of pH, glucose and protein metabolism, maintenance of the normal structure, integrity and function of the intestines (Ríos-Covián et al., 2016; Markowiak-Kopeć and Śliżewska, 2020). These fatty acids have been also recognized as potential mediators involved in the effects of gut microbiota on intestinal immune function, showing anti-inflammatory activity (Markowiak-Kopeć and Śliżewska, 2020). Moreover, studies in animals and humans revealed that gut microbiota dysbiosis has been implicated in behavioural and neurologic pathologies, supporting the SCFAs' crucial physiological effects on several organs, including the brain (Sharon et al., 2016; Stilling et al., 2016; Fung et al., 2017; Deng et al., 2019). Ageing is associated with an alteration of intestinal balance and a progressively and significant reduction in the faecal concentrations of SCFAs. Since the maintenance of intestinal homeostasis is necessary to maintain the host's well-being and prevent many age-related diseases, the use of probiotics is one of the most helpful strategies to modulate gut microbiome and SCFAs, improving elderly people health (Dinan and Cryan, 2017; Markowiak-Kopeć and Śliżewska, 2020). This study aimed to evaluate the effects of probiotic dietary supplementation on SCFAs level production in healthy elderly people. At baseline conditions, despite the high variability between different genders and residence structures, no significant variations were observed. After dietary intervention, the main changes were referred to the most abundant SCFAs, acetic and butyric acids, with a greater increase in SCFAs level in probiotic group than placebo one. The same trend was observed for total SCFAs, with an evident but not significant increase in acids amount after probiotic supplementation. The other SCFAs did not show any change and remained stable after intervention period. Considering the SCFAs' key role

and their physiological age-related reductions, the increase in SCFAs' levels obtained in this study are a very interesting result, that supports, once again, the use of probiotics as a potential strategy to modulate elderly health.

5.2 INTRODUCTION

Human mucosal sites are colonized by a huge number of microorganisms of different kingdoms and genera. Most of these cells are located in the gastrointestinal (GI) tract where they exert several roles, such as protective, structural and metabolic functions (O'Hara and Shanahan, 2006). Relevant effects of these microorganisms have been demonstrated not only in the GI tract but also in adipose tissue, immune and nervous systems (Maslowski et al., 2009; Vijay-Kumar et al., 2010; Heijtz et al., 2011). Moreover, studies revealed that not only gut microbiota, but also its metabolites like short chain fatty acids (SCFAs), are involved in many pathways, suggesting their role in the development of pathological conditions including inflammatory bowel disease (IBD), colon cancer, obesity and type 1 and 2 diabetes mellitus (Maslowski et al., 2009; Uronis et al., 2009; De Filippo et al., 2010; Vijay-Kumar et al., 2010). Throughout life, with ageing and other factors, several modifications in the gut microbiota and, consequently, in its products amount occur, and this can strongly influence the energy balance of the host, especially in elderly people (Markowiak-Kopeć and Śliżewska, 2020). SCFAs are the main metabolites produced in the colon by bacterial fermentation of dietary fibres and resistant starch (Markowiak-Kopeć and Śliżewska, 2020). SCFAs play a very important role in regulating pH, increasing the absorption of calcium, iron, as well as magnesium, and are beneficial for glucose and protein metabolism in the liver. In addition, these acids affect the maintenance of the normal structure, integrity and function of the intestines (Ríos-Covián et al., 2016; Markowiak-Kopeć and Śliżewska, 2020). They show also anti-inflammatory activity, which involves the inhibition of the activity of inflammatory mediators in the intestinal epithelium, inhibiting, for example, the activation of NF-kB macrophages, which are the main source of cytokines in case of inflammatory processes (Markowiak-Kopeć and Śliżewska, 2020). Moreover, growing evidences support the SCFAs' crucial physiological effects on several organs, including the brain (Stilling et al., 2016; Fung et al., 2017). This hypothesis is supported by studies in animals and humans showing that gut microbiota dysbiosis has been implicated in behavioural and neurologic pathologies, such as depression, Alzheimer's (AD) and Parkinson's (PD) diseases and autism spectrum disorder (ASD) (Sharon et al., 2016; Deng et al., 2019). Considering the direct connection between gut microbiota and SCFAs, the modulation of microbiota and

microbiome in elderly people could be a reliable strategy to improve the geriatric condition and all the age-related diseases (Dinan and Cryan, 2017). In this study we aimed to evaluate the effects of 6-month probiotic supplementation on SCFAs level in faecal sample of healthy elderly people.

5.3 MATERIALS AND METHODS

The SCFAs analysis have been performed with the collaborations of Prof. Dennis Fiorini's team.

5.3.1 Standards, reagents and solvents

The analytical standards, acetic, propionic, *i*-butyric, *n*-butyric, *i*-valeric, *n*-valeric, *i*-caproic and *n*-caproic acids (C2, C3, *i*C4, C4, *i*C5, C5, *i*C6 and C6 respectively), were acquired from Sigma–Aldrich (Milan, Italy). Sulfuric acid was purchased from Carlo Erba (Milan, Italy) and ethyl ether from J.T. Baker (Phillipsburg, New Jersey, USA).

5.3.2 Standard solutions preparation

For each acid, a diethyl ether stock standard solution was prepared. The concentration was 7.00×10^3 µM for the most abundant SCFAs (C2, C3 and C4) and 1.00×10^3 µM for the less abundant SCFAs (*i*C4, *i*C5, C5 and C6). The stock standard solutions for the two internal standards were prepared dissolving 24 µL of *i*C6 (IS1) and 120 µL of C5 (IS2) in diethyl ether (10 mL for each solution). All the stock standard solutions were stored at 4 °C until used.

5.3.3 SCFAs extraction

The volunteers' faecal samples were collected and delivered to the University, where they have been immediately frozen at -20°C until the analysis. After homogenization, an aliquot of 100 mg of sample was weighted in a 2 mL vial, acidified with 0.25 mL of aqueous sulfuric acid 50% w/v and shaken for 3 min. The internal standard solution (IS1) was added (50 µL of *i*C6) and an extraction with 1 mL of ethyl ether was performed. After 5 min centrifugation at 2800 x g, the organic phase was collected into a 4 mL vial. The extraction procedure was repeated three times up to collect 3 mL of organic phase in total. At the end, 0.5 µL of the solution were injected into the GC for the analysis.

5.3.4 GC-FID analysis of SCFAs

The analysis was performed using a gas chromatograph Agilent Technologies 6850 GC (Santa Clara, CA, USA) associated with a split/spitless injector and FID. To confirm the identity of the analytes a GC 6890N combined with a mass spectrometer detector 5973 (Agilent Technologies, Santa Clara, CA, USA) was also used. The capillary column was a nitroterephthalic acid modified polyethyleneglycol (PEG) column (DBFFAP, 25 m, 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA). The GC injector was set at 280 °C and the injection was performed in splitless mode (splitless time: 3 min). The oven temperature started from 40 °C for 3 min, then increased of 20 °C/min to 160°C and finally of 40 °C/min until 245 °C and maintained for 1.87 min, resulting in a total run time of 13 min. Hydrogen was used as carrier gas at a flow rate of 3.70 mL/min. The detector temperature was maintained at 250 °C. MS operational parameters were: electron ionization (EI) at 70 eV; transfer line and ion source temperature: 250 °C; quadrupole temperature: 150 °C; and mass range: *m/z* 29–300. The SCFAs identity in real samples was confirmed by comparison of their retention times and their mass spectra with those of authentic standards and with reference spectra from the US National Institute of Standards and Technology (NIST, 2008) (Scortichini et al., 2020).

5.3.5 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) and Tukey's test for pairwise comparison, in order to determine significant differences ($P<0.05$) between the different number of extractions applied to the samples, using the software PAST(Hammer et al., 2001).

5.4 RESULTS

5.4.1 SCFAs quantitative determination at baseline

The faecal SCFAs quantification was performed at two different time points, before and after the intervention, to assess the baseline conditions and monitor potential changes during time. The results of SCFAs analysis are presented in Figure 1. At baseline conditions, there is a high inter-individual variability in each group, and between different groups or gender. In general, acetic, propionic and butyric acids are the most abundant, while isobutyric, valeric and caproic acids are present in less amount. Figure 1 shows the

single SCFAs level considering the gender and the residence structures. Despite the high variability, there are not statistically significant differences between the compared groups.

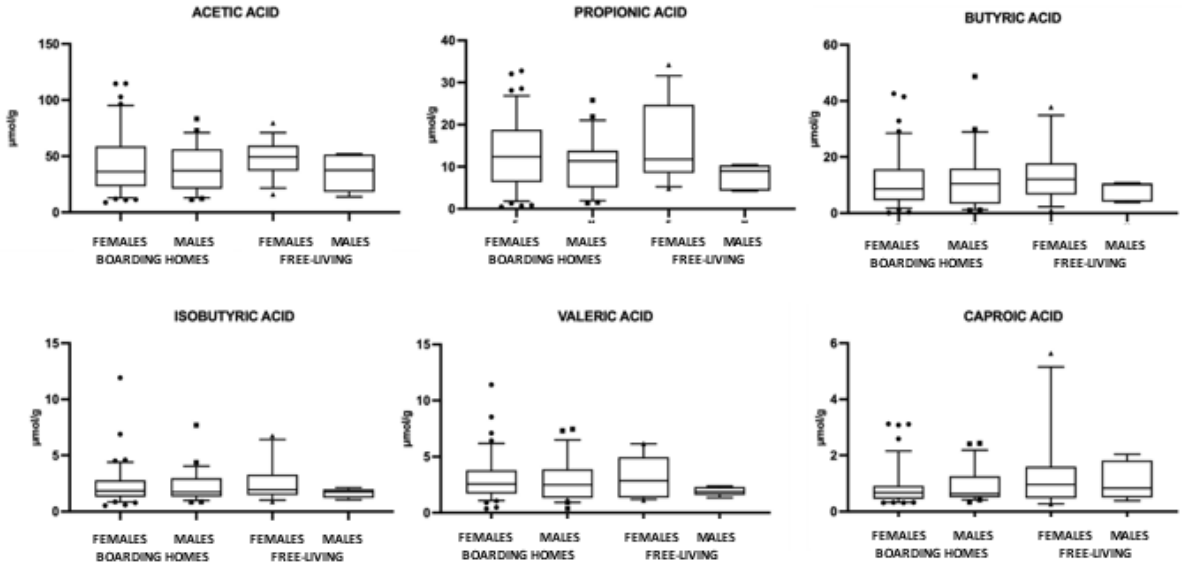

Figure 1. Faecal short chain fatty acids at baseline conditions, according to the gender and residence structures. Values expressed as µmol/g of faeces (n=97).

5.4.2 SCFAs quantitative determination after intervention

The Figure 2 shows the single and total faecal SCFAs levels in placebo and probiotic groups after the dietary intervention. Analysing acetic, propionic and butyric acids, the most abundant SCFAs, we observed some variations between time T0 and time T1 within the same group (placebo or probiotic). The major changes, although not significant, were referred to acetic and butyric acids after probiotic supplementation, with an increase in concentration in both cases. A similar trend was observed in placebo group, with slight changes after the intervention. The levels of the other SCFAs, present in smaller quantities, remained mainly unchanged under 5 µmol/g of faeces and no statistically significant differences were noticed. Moreover, studying the total SCFAs level, the probiotic group had an evident increase in SCFAs concentration at the end of the intervention period respect to T0; the increment was slightly higher than placebo group ($P>0.05$).

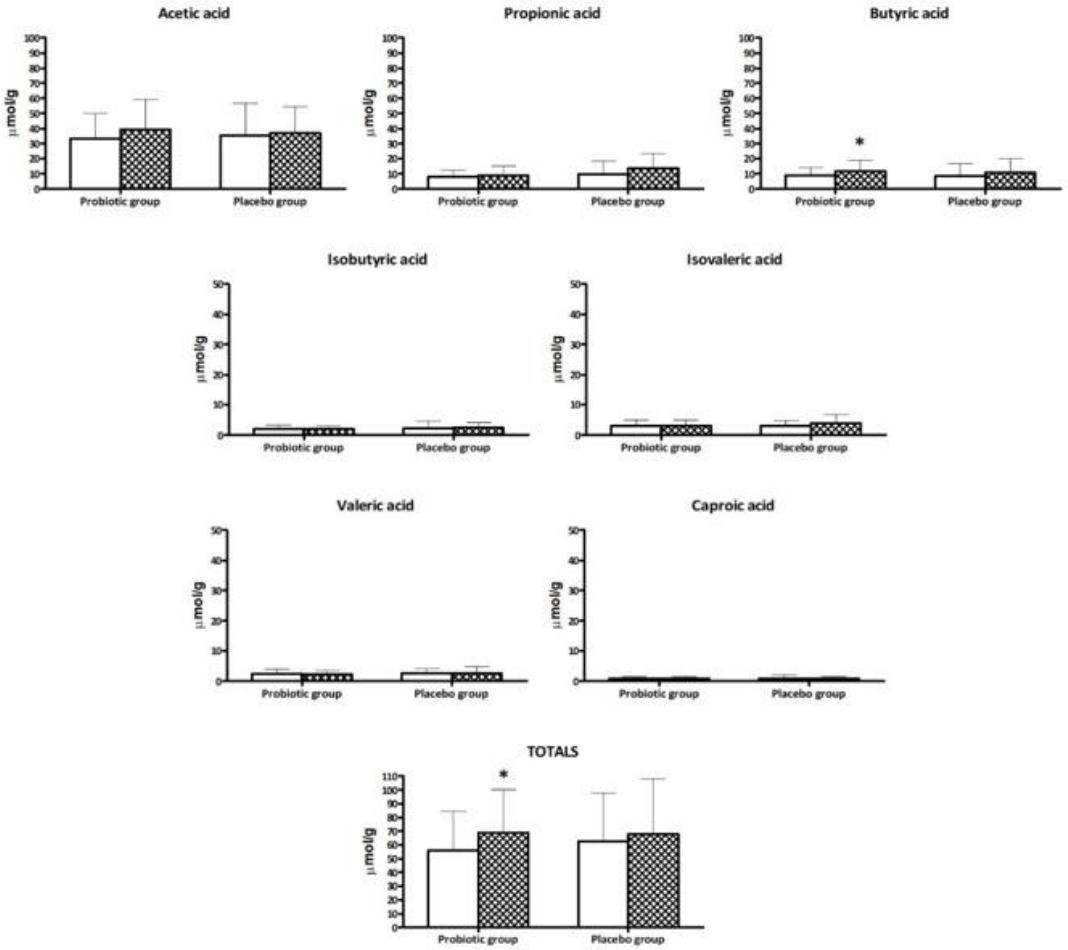

Figure 2. Single and total SCFAs levels during the different time points (▭ T0 and ▩ T1) relative to the probiotic and placebo group.

To explore in detail the changes in SCFAs levels after the intervention, we further divided the subjects into four groups, basing on the type of supplementation received and the residence structure. According with the first elaboration presented in Figure 2, the main variations were observed for acetic and butyric acids. Figure 3 shows the differences in SCFAs levels between free-living and boarding home subjects; at time T1 evident but not significant increases were observed respect to T0, despite the treatment. While, taking into account the type of supplementation and its effect on SCFAs, at time T1 probiotic group had a greater increase than the placebo one. On the contrary, for the less abundant SCFAs, not significant differences were reported considering the type of supplementation and the residence structures (Figure 3).

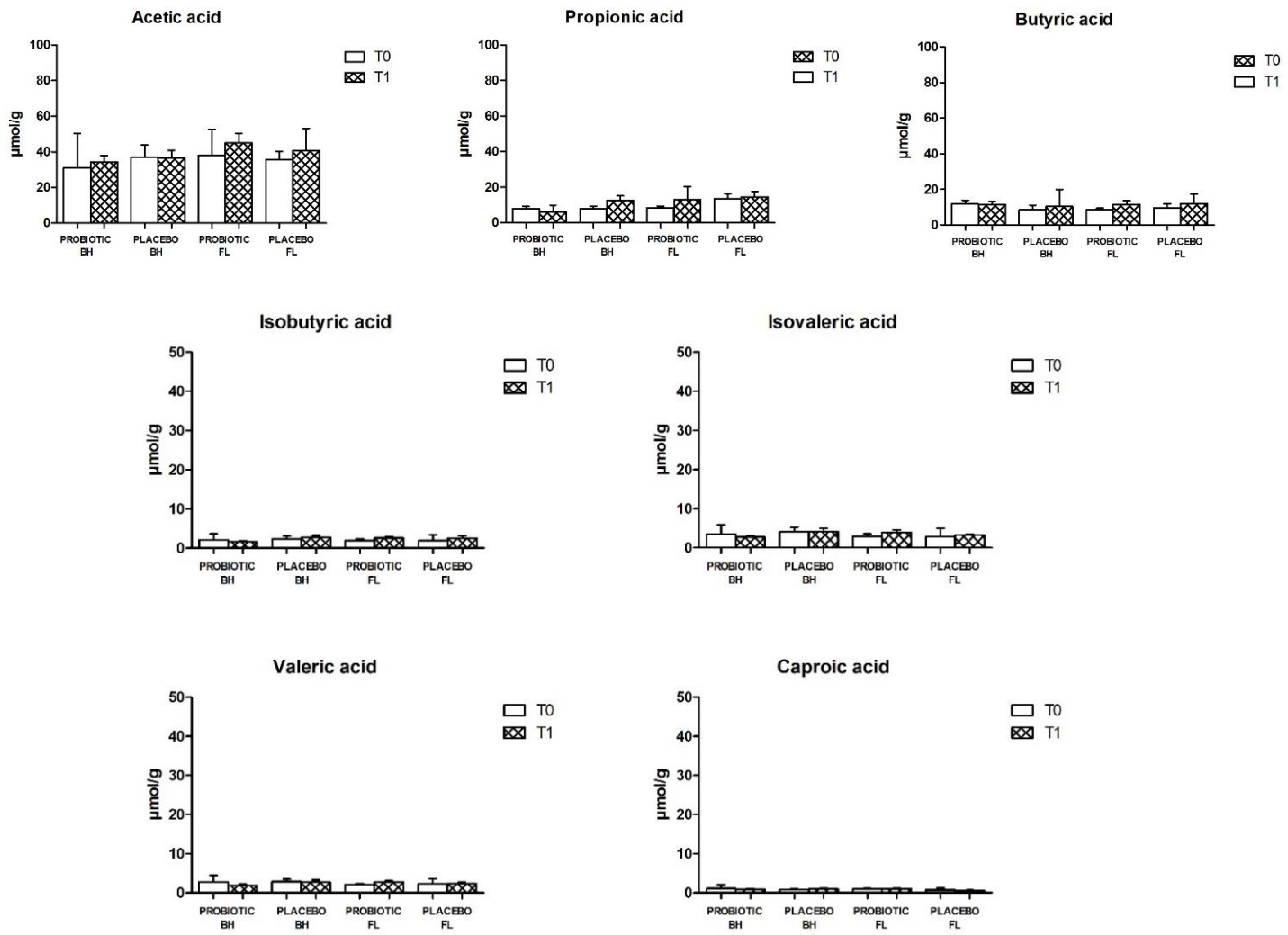

Figure 3. Single SCFAs levels during the different time points relative to the type of supplementation and residence structure (BH: boarding homes; FL: free-living).

5.5 DISCUSSION AND CONCLUSIONS

Several studies demonstrated the alteration of gut microbiota composition in elderly people, with increased levels of some bacterial groups at the expense of others (Hopkins et al., 2001; Woodmansey, 2007; Biagi et al., 2010, 2016; Gueimonde et al., 2010; Mäkivuokko et al., 2010; Salazar et al., 2013). Maintaining the balance of intestinal microbiome is crucial for maintaining the normal health status, especially in elderly people (Markowiak-Kopeć and Śliżewska, 2020). The age-related changes in gut microbiota composition lead also to a direct reduction in the intestinal levels of SCFAs (Salazar et al., 2013).

SCFAs, metabolites of intestinal bacteria, exert many important functions and they are influenced by the composition of GI microbiota, genetic, environmental and dietary factors (Markowiak-Kopeć and Śliżewska, 2020).

Since the alterations of intestinal microbiota and SCFAs production play an essential role in triggering inflammatory events, such as impairment of intestinal barrier integrity and gut leakiness increase, endotoxemia, inflammageing and associated morbidities, the modulation of GI microbiota and SCFAs using probiotics could be useful to improve the age-related condition (Rera et al., 2012; Clark et al., 2015). This part of the study aimed to evaluate the effects of a probiotics-based diet on SCFAs produced by intestinal microbiota in clinically healthy older people. The faecal SCFAs quantification was performed at two different time points, before and after the intervention. At baseline conditions, a high inter-individual variability in each group, and between different groups or gender was observed. In general, acetic, propionic and butyric acids are the most abundant, while isobutyric, valeric and caproic acids are present in less amount. The major findings were observed at T1 in probiotic group, with an increase in concentration for acetic and butyric acids, although not significant. The levels of the other SCFAs, present in smaller quantities, remained mainly unchanged in probiotic and placebo groups. In addition, analysing total SCFAs level, in probiotic group an evident increase in SCFAs concentration at T1 respect to T0 was noticed, slightly higher than placebo group ($P>0.05$). After a further subgroup analysis, no significant variations were observed in SCFAs levels taking into account just the residence structure.

Contrary to the expected progressive reduction of SCFAs in the GI tract associated with ageing, in this study we observed the opposite behaviour (Silva et al., 2020). Despite some limitations and the lack of significance, an evident increase in the amount of the most abundant SCFAs was detected after probiotic dietary intervention. On the contrary, the minor SCFAs mainly maintained the baseline concentrations in all elderly volunteers. One of the limitations of this study is the measurement of SCFAs production that was performed in faeces. This analysis reflects their levels at the end of the digestive tract, but not necessarily it describes the other parts of the colon.

Considering all these aspects, the key role played by the intestinal SCFAs and the serious consequences in terms of gut barrier maintenance and host physiological homeostasis caused by age-related reductions in SCFAs, the results obtained in this study

are very interesting and the probiotics use seems to be a right direction for further research (Ríos-Covián et al., 2016).

5.6 REFERENCES

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CHAPTER VI

MONITORING OF BIOGENIC AMINES ON PROBIOTICS-SUPPLEMENTED SENIORS

6.1 ABSTRACT

Biogenic amines (BAs) are biologically active molecules present in different districts of human body, exerting several essential physiological functions. Since BAs can be used as markers of different and important diseases, their detection and quantification in biological fluids and tissues can be useful to evaluate the health status of people. In this study, the level of 11 BAs was determined in urine samples to assess the effects of probiotics supplementation on healthy elderly volunteers. The results revealed a high inter and intra-variability at baseline and at the end of dietary intervention. Polyamines: spermidine levels positively decreased in probiotic group, remaining stable in placebo group at T1; putrescine and spermine decreased in both groups, despite the supplementation; on the contrary, cadaverine increased in probiotic group, decreasing in placebo group. Histamine and dopamine decreased after both supplementations; serotonin levels remained mainly unchanged in probiotic group respect to placebo; the opposite trend for tyramine amount that decreased. Phenylethylamine increased after probiotic and placebo treatments. On the contrary, epinephrine and trymetilamine were not detected in the urine samples, because of their absence or their presence in quantities out of LOQ (limit of quantification) and LOD (limit of detection) ranges. Urine test proved to be inexpensive, quick, and non-invasive, despite some limitations. We concluded that the effect of probiotic supplementation was demonstrated as a trend influencing some BAs more than others. To investigate elderly health status and the influence on age-related diseases, more studies are needed to better understand the potential mechanisms by which probiotics modulate BAs, and consequently healthy ageing.

6.2 INTRODUCTION

Scientific opinion and interest are becoming more aware about the importance of biogenic amines (BAs) for human health (Gosetti et al., 2013). The most attention in research on BAs is referred to their presence in some fermented foods and beverages, where they naturally are formed during fermentation processes from amino acid decarboxylation (Ten Brink et al., 1990; Silla Santos, 1996). BAs present in foods are indicators of food quality and potentially toxic to human health when ingested in large amount (Lehane and Olley, 2000; Ancín-Azpilicueta et al., 2008; Spano et al., 2010).

Recently, the interest has also shifted towards the presence of BAs in biologic fluids and tissues, as many correlations with human health have been demonstrated (Gosetti et al., 2013).

Biogenic amines are low molecular weight organic nitrogen compounds formed by the decarboxylation of amino acids or amination and transamination of aldehydes and ketones during the standard metabolic processes (Gosetti et al., 2013; Erdag et al., 2018). BAs are biologically active molecules present in different districts of human body, exerting several essential physiological functions (Erdag et al., 2018). In general, BAs are source of nitrogen and precursors for the synthesis of important compounds in organisms, such as alkaloid, nucleic acids, hormones, and proteins, which derived from the normal metabolism in animals, plants and microbial cell (Novella-Rodríguez et al., 2002; Latorre-Moratalla et al., 2012). Moreover, BAs have biological functions in human immune system, nervous system as neurotransmitters, and cardiovascular system, increasing or decreasing blood pressure; they also play an important role in metabolism and digestion (Erdag et al., 2018).

The wide world of BAs includes two main groups, polyamines (PAs) and catecholamines (CATs), both with specific roles in human physiology (Gosetti et al., 2013). In addition, according to their chemical structures, BAs can be classified in three groups as follow: monoamines (Tyramine, Phenylethylamine), diamines (Histamine, Serotonin, Putrescine, Cadaverine) and polyamines (Spermidine, Spermine, Agmatine).

Polyamines (PAs) are widely spread in human body and they are involved in cancer cell growth (Khuhawar and Qureshi, 2001). For some PAs, like Spermine (Spm), Spermidine (Spd), Putrescine (Put), and Cadaverine (Cad), studies demonstrated that their

concentration significantly increases in biological fluids and tissues of cancer patients (Khuhawar and Qureshi, 2001; Lozanov et al., 2007). Although the metabolism pathway has not been clearly interpreted yet, high levels of total PAs, as observed in cancer patients, have been associated with altered PAs biosynthesis and accumulation, and consequent rapid tumour growth. Positively, the PAs concentration could be useful, with the medical history of the patient, in the diagnosis of this kind of disease (Khuhawar and Qureshi, 2001; Lozanov et al., 2007; Gosetti et al., 2013).

However, catecholamines (CATs) derive from a common precursor, the amino acid tyrosine and they include Serotonin (5-HT), Dopamine (DA), and Epinephrine (E). CATs are natural endogenous molecules that act as hormones and neurotransmitters in the central and peripheral nervous systems (Nagatsu, 2006; Baumann et al., 2009; Gosetti et al., 2013). CATs are involved in the regulation of the response to stress psychomotor activity, emotional processes, learning, sleep, memory (Gosetti et al., 2013). According to these evidences, urinary and plasmatic CATs have been proposed as stress biomarkers (Ray et al., 2006; Mitoma et al., 2008). CATs have been also suggested as growth factors and antioxidants, since they participate in cell multiplication and regulation processes. Their detection in body fluids is important for clinical diagnosis of pheochromocytoma, paraganglioma, and neuroblastoma, neuroendocrine disorders and other physiologic and pathologic conditions (Chan and Ho, 2000; Kushnir et al., 2002; Marc et al., 2011).

Each BA is involved in specific pathways related with human physiology. For example, DA is related with locomotion, cognition and development, and gut motility (Esler et al., 1990; Civelli et al., 1993; Brown et al., 2001; Carrasco and Van de Kar, 2003); acetylcholine (Ach) is a neurotransmitter that transfers action potentials between neurons to communicate with specialized cells such as muscles; 5-HT is part of the regulation of many physiological functions such as mood stabilization, appetite regulation, digestion, sleep, sexual behaviour, cerebral blood flow regulation, and blood-brain barrier permeability (Nakada and Itoh, 2003); histamine (His) is involved in local immune reactions and related inflammatory responses, in regulating body temperature, stomach volume and pH (Nieto-Alamilla et al., 2016); tryptamine (Tryp) is found in trace levels in the brain of mammals, it increases blood pressure and acts as neuromodulator and neurotransmitter (Berry, 2004).

Among all BAs, the amines with function as neurotransmitters include DA, norepinephrine (NE), E, His, and 5-HT. In addition to the well-established roles as neurotransmitters, accumulating evidences suggest that these amines might act in the gut as important signal molecules between commensal microbiota and the host (Freestone et al., 2008; Hughes and Sperandio, 2008; Lyte, 2004). It has been hypothesised the potential role of microbe-derived amines and other trace amines in modulating the gut physiology but also the host brain functions, the "microbiota-gut-brain" axis (Cryan and Dinan, 2012; Schmidt, 2015).

Recent research also evidenced the potential involvement of biogenic amines in muscular system function changes. He and Jasper (2014) studied the correlation between ageing and progressive decline in muscular integrity and function. Despite the increasing studies regarding age-related muscular system function alterations, the mechanisms behind these changes remain unclear. One of the possible underlying mechanisms could be referred to biogenic amines, since age-related changes in their level have been reported, especially for serotonin and dopamine that vary with ageing (Seid and Traniello, 2005; Rauschenbach et al., 2011).

Since it was demonstrated that BAs can be markers of different and important diseases, the simultaneous determination of BAs in biological fluids and tissues can be useful to develop a BAs profile for clinical diagnosis medicine.

This study aimed to monitor the effects of probiotic functional foods-based diet on health in elderly people. According to the evidence, the biogenic amines levels analysis is a valid approach to investigate health status in elderly and the incidence of age-related diseases. Despite the existing limitation regarding the bioamines' low concentrations in biological fluids, undoubtedly, for patients, a urine test is rapid, simple, and less invasive than others. In this study eleven biogenic amines have been detected and quantified in urine samples of healthy elderly people before and after the dietary intervention, with the aim to monitor any variations according with probiotic/placebo supplementation.

6.3 MATERIALS AND METHODS

The BAs analysis have been performed thanks to the collaboration of Prof. Gianni Sagratini's team.

6.3.1 Chemicals and Reagents

Perchloric acid ($HClO_4$, 80%), acetone (≥99.5% CAS No. 67-64-1), sodium hydroxide anhydrous (≥98%, CAS No.1310-73-2), sodium bicarbonate anhydrous (≥99.5%, CAS No. 144-55-8), acetonitrile (HPLC, gradient grade, ≥99.9%, CAS No. 75-05-8) and dansyl chloride ($C_{12}H_{12}ClNO_2S$, 98% CAS No. 605-65-2) for deproteinization, extraction and derivatization were from Sigma-Aldrich (Milano, Italy). Deionized water (<8MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

6.3.2 Standard and solutions

Spermine tetrahydrochloride (SPE, $C_{10}H_{26}N_4 \bullet 4HCl$, >98%, CAS No. 306-67-2), Spermidine trihydrochloride (SPD, $C_7H_{17}N_3 \bullet 3HCl$, >98%, CAS No. 334-50-9), Cadaverine dihydrochloride (CAD, $C_5H_{14}N_2 \bullet 2HCl$, >98%, CAS No. 1476-39-7), Putrescine dihydrochloride (PUT, $C_4H_{12}N_2 \bullet 2HCl$, >98%, CAS No. 333-93-7), Histamine dihydrochloride (HIS, $C_5H_9N_3 \bullet 2HCl$, >99%, CAS No. 56-92-8), Tyramine hydrochloride (TYR, $C_8H_{11}NO \bullet HCl$, >98%, CAS No. 60-19-5), 2-Phenylethylamine hydrochloride (PEA, $C_8H_{11}N \bullet HCl$, >98%, CAS No. 156-28-5), Tryptamine hydrochloride (TRY, $C_{10}H_{12}N_2 \bullet HCl$, >99%, CAS No. 343-94-2), (-)-Epinephrine (E, $C_9H_{13}NO_3$, CAS No. 51-43-4), Dopamine hydrochloride (DA, $(HO)_2C_6H_3CH_2CH_2NH_2 \bullet HCl$, 98%, CAS No. 62-31-7) and Serotonin (5-HT, $C_{10}H_{12}N_2O$, >98%, CAS No. 50-67-9) for standard solutions preparation were supplied by Sigma-Aldrich (Milano, Italy). Individual stock solutions of BAs (1000 mg/L) were prepared by dissolving 10 mg of each compound in 10 mL of HCl 0.1 M (Merck Darmstadt, Germany). Standard working solutions, at various concentrations, were daily prepared by stock solutions with HCl 0.1 M. Internal standard (IS) solution was prepared at 10 mg/mL in HCl 0.1 M. Solutions were stored at 4 °C until used.

6.3.3 Urine samples collection and processing

In the present study urine samples of 97 volunteers living in boarding homes and in private houses were collected and frozen at −80 °C within 1 h after collection until the day of analysis. The biological samples were collected at two different time points, at baseline (T0) and after the supplementation with probiotic or placebo (T1). The preparation of the samples was realized through three phases: deproteinization, derivatization and purification.

6.3.4 HPLC-FLD analysis

BAs detection and quantification were performed using a HPLC Agilent 1260. The chromatographic conditions used were flow rate of 0.8 mL min^{-1}, volume of injection of 20 µL and column temperature of 40°C. Samples were separated on a Kinetex C18 2.6µ 100A 100x4.6mm column. Different mobile phases and gradients were tested to better separate the amines. The HPLC instrument was coupled with an Agilent Fluorescence Detector. In order to establish the Fluorescence Detector (FLD) acquisition wavelength, a multi-excitation analysis and a multi-emission analysis were performed the first in the range of 200 - 400 nm and the second in the range of 400 – 700 nm.

6.3.5 Statistical analysis

Data are expressed in µg/L and were analysed by Tukey's Multiple Comparison Test (ANOVA) and Student's T test to detect the differences in BAs levels after the intervention. Statistical significance was considered when the probability value $P < 0.05$.

6.4 RESULTS

6.4.1 Extraction and HPLC-FLD method optimization

The quantitative analysis of biogenic amines in urine, like in other complex matrices, needs a special attention on the sample preparation and cleaning up procedure in order to obtain complete extraction of the analysed components and removal of proteins and sample components. In this study, the technique used was the same adopted by Lozanov *et al.* (2007) to extract and precipitate proteins during the simultaneous analysis of amino acids and biogenic amines in biological fluid.

For HPLC-FLD method optimization, different mobile phase systems including acetonitrile–water and methanol–water in various proportions were tested. Lower background noise and better symmetric peaks were obtained by Milli-Q H2O (Solvent A) and MeOH:CH_3CN 70:30 v/v (Solvent B). The gradient was optimised as follow to better separate the eleven amines: 0 min 60% B, 10 min 70% B, 20 min 80% B, 26 min 100% B, 29 min 100% B and 32 min 60% B until 36 min, the analysis time. Under these conditions, BAs were well separated in a Kinetex C18 2.6µ 100A 100x4.6mm column. Calibration curves were obtained by injection of 6 different concentrations of BAs standard solutions (37.5, 75, 156.25, 312.5, 625 and 1250 µg/L) with the IS and calculating the response

factor (BA peak area/IS peak area). The linear regression coefficient of all calibration curves was (R2) higher than 0.990. RSD% values underlined the method precision, by that were all under 20%. Sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ) values. LODs values were from 1.4 µg/L to 18.75 µg/L and LOQs ranged from 5.63 µg/L to 37.5 µg/L, and these values are in line with recent literature (Table 1) (Liang et al., 2004; Gosetti et al., 2013; Naccarato et al., 2014; Maráková et al., 2020).

Table 1. Limit of quantification (LOQ) and Limit of detection (LOD) for each biogenic amine analysed.

BAs	Biogenic Amines	LOQ (µg/L)	LOD (µg/L)
Put	*Putrescine*	7.50	2.81
Cad	*Cadaverine*	5.63	2.81
Spd	*Spermidine*	7.50	2.81
Spm	*Spermine*	5.63	2.81
Pea	*2-Phenylethylamine*	7.50	5.63
His	*Histamine*	37.50	9.38
5-HT	*Serotonin*	37.50	9.38
Tyr	*Tyramine*	9.38	7.50
DA	*Dopamine*	75.00	37.50
Tryp	*Tryptamine*	9.38	5.63
E	*Epinephrine*	37.50	18.75

6.4.2 Biogenic amines quantitative determination at baseline

In this study, the method used allowed the quantification of 11 BAs at the same time, in each urine sample. BAs concentrations were detected at baseline and after the probiotic/placebo intervention.

The data at baseline conditions are presented in Table 2, where the range of concentration values for each single and for total BAs is shown. As expected from a biological matrix as urine, the individual results reported a great variability among subjects. The data have been organized according to the residence structure into boarding home and free-living subjects. For each BA, and for the total amount, the detected minimum and maximum value in the two groups are indicated. According to Table 1, the quantification of specific BAs in some samples was not achievable, due to the absence of the compound or to the fact that the values detected are out of the ranges of quantification and detection.

Cad, Spm, His, 5-HT and DA were detected in some urine samples, as reported in Table 2. For each group of subjects, the frequency (reported as %) of null value for the BA, varies from 2.9% (in BH subjects) to 64.7% (in FL subjects), but in relation of different BA, Spd in first case and DA in the second. Table 2 highlights a wide variability of values for each single BA within each group, describing in general higher BAs concentrations in BH than FL subjects.

Four BAs were present with detectable value in all analysed samples: Pea, Put, Tyr, and Spd, although the latter in free-living subjects only. This situation could be due to the higher concentrations of these BAs in comparison with the others in human urine and to the lower values of LOQ, that corresponds to a higher sensitivity of the method for these amines.

On the contrary, Tryp and E were not detected in any samples. One of the reasons could be the low concentrations or absence of Tryp in human urine, according to the results of Maráková *et al.* (Maráková et al., 2020). E could be present in human urine but with the method used in this analysis, it showed the highest value of LOQ, so it was not quantifiable under 37.5 µg/L.

Table 2. Concentrations of single and total BAs detected in urine samples of boarding home and free-living subjects at T0. For each BA, the minimum and maximum values are reported (µg/L). The frequency of subjects in which the BA were not detected, or BA values were > LOD but < LOQ, is expressed as percentage. BH= boarding home; FL = free-living.

Biological amine	BH SUBJECTS		FL SUBJECTS	
	MIN-MAX (µg/L)	Frequency of null value	MIN-MAX (µg/L)	Frequency of null value
Put	29.4 – 6131.4	0%	10.3 – 363.6	0%
Cad	5.8 – 1774.7	17.6%	11.2 – 271.6	17.6%
Spd	8.5 – 546.7	2.9%	7.9 – 1193.3	0%
Spm	8.2 – 1424.6	11.8%	7.1 – 614.8	17.6%
Pea	244.5 – 14169.6	0%	122.1 – 5207.3	0%
His	95.5 – 9427.3	11.8%	153.6 – 2184.1	5.9%
5-HT	66.2 – 1284.2	73.5%	(a)	100%
Tyr	30.5 – 5796.8	0%	31.7 – 839.6	0%
DA	147.5 – 9298.1	35.3%	152.6 – 441.6	64.7%
Tryp	(a)	100%	(a)	100%
E	(a)	100%	(a)	100%
TOTAL BAs	**1036 – 22899.7**		**357.3 – 7464**	

(a) - null value: BA value not detected, or BA values were > LOD but < LOQ.

6.4.3 Biogenic amines quantitative determination after intervention

At the end of the intervention, BAs analysis was repeated to evaluate the changes respect to the baseline profile of probiotic and placebo groups.

Table 3 shows the concentrations of single and total BAs detected in urine samples of placebo and probiotic group at T1. For each BA, the minimum and maximum values are also reported (μg/L). The frequency of subjects in which the BA were not detected, or BA values were > LOD but < LOQ, is expressed as percentage.

Pea and Tyr were detected in the analysed samples of all of subjects. Their detection could be related to their high concentration in urine samples, or the high sensitivity of the method used for these amines.

Put, Cad, Spm, His, 5-HT, Spd and DA were not detected in all urine samples, each group showed a variable percentage of not detected BAs, due to their absence or their values out of LOD and LOQ ranges.

As observed at baseline, Tryp and E were not detected in any of the samples at T1. These amines could be absent in the analysed urine samples or out of the detection and quantification ranges, as reported in Table 1 (Maráková et al., 2020).

The high intra- and inter-variability in each group shown at T0 is confirmed after the dietary intervention (T1). In details, Figure 1 shows the group of polyamines, a group of BAs comprising Put, Cad, Spd and Spm. These BAs, widely spread in the human body, are related with cancer cell growth. Put and Spm levels decreased at T1 respect to T0 in probiotic and placebo groups, although with no statistical significance. Spd had an evident decrease in probiotic group, while in placebo group the level remained unchanged. Probiotic group showed a non-significant increase in Cad concentration at T1; on the contrary, placebo group had a decrease in Cad level.

Figures 2 shows the levels of Histamine, Serotonin, Tyramine and Dopamine, they are considered in group because they act as important messengers and regulators of several cell functions, including locomotion, cognition, and development. His and DA decreased the level in either probiotic or placebo groups from T0 to T1. Probiotic group had a decrease in Tyr level respect to placebo one, where it remained stable during time. 5-HT levels were detected in few urine samples (about 90% of sample was null); considering the detected concentrations in probiotic group at T0 and T1, they were approximately the same. On the contrary, a decrease was observed in placebo group (Figure 2) at T1. 2-

phenylethylamine levels are presented in Figure 3, where a common increasing trend is observed after probiotic and placebo interventions. Figure 4 describes the variation in BAs total amount, obtained from the mean values of single amines. In this case, an opposite trend between probiotic and placebo groups is observed. The total BAs level decreased after probiotic intervention, while the level increased in placebo group. In both cases, the variations are not statistically significant, probably due to the high variability between biogenic amines observed in both groups (Figure 4).

Table 3. Concentrations of single and total BAs detected in urine samples of Placebo and Probiotic group at T1. For each BA, the minimum and maximum values are reported (µg/L). The frequency of subjects in which the BA were not detected, or BA values were > LOD but < LOQ, is expressed as percentage.

Biological amine	PLACEBO GROUP		PROBIOTIC GROUP	
	MIN-MAX (µg/L)	Frequency of null value	MIN-MAX (µg/L)	Frequency of null value
Put	15.30 – 1761.02	10.5%	7.3 – 3507.0	3.1%
Cad	17.5 – 570.8	31.6%	7.38 – 2508.8	28.1%
Spd	8.03 – 209.9	10.5%	11.6 – 451.9	12.5%
Spm	6.1 – 481.3	26.3%	5.7 – 427.2	21.9%
Pea	193.6 – 26205.9	0%	211.1 – 11832.0	0%
His	104.34 – 7450.2	36.8%	20.21 – 2124.4	43.8%
5-HT	73.1 – 195.1	89.5%	152.15 – 311.09	87.5%
Tyr	36.0 – 408.7	0%	10.6 – 610.0	0%
DA	103.5 – 981.0	68.4%	60.4 – 774.2	62.5%
Tryp	(a)	100%	(a)	100%
E	(a)	100%	(a)	100%
TOTAL BAs	**506.5 – 33924.4**		**786.5 – 12667.5**	

(a) - null value: BA value not detected, or BA values were > LOD but < LOQ.

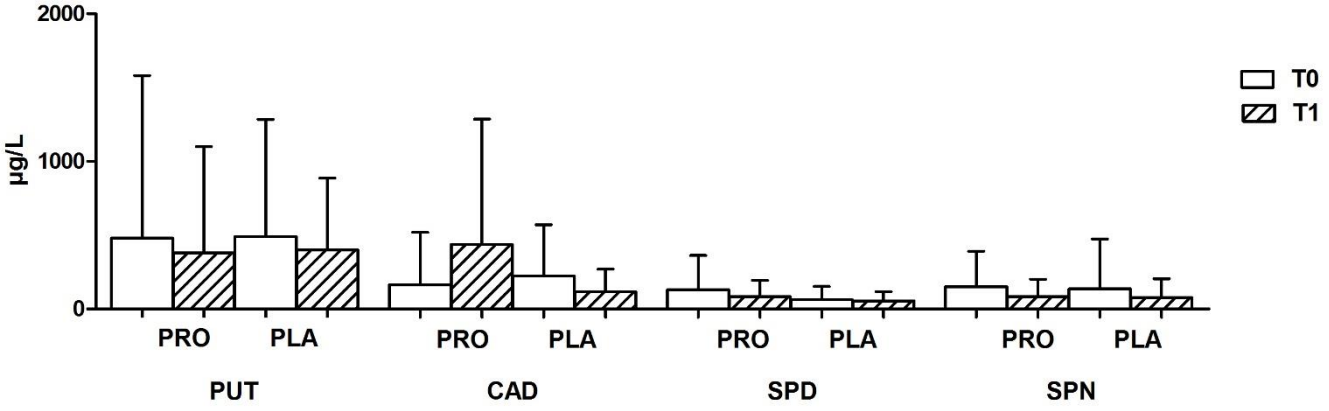

Figure 1. Putrescine, Cadaverine, Spermidine and Spermine concentrations in urine samples of placebo and probiotic supplemented groups at T0 and T1. The results are expressed as mean values ± SD (µg/L).

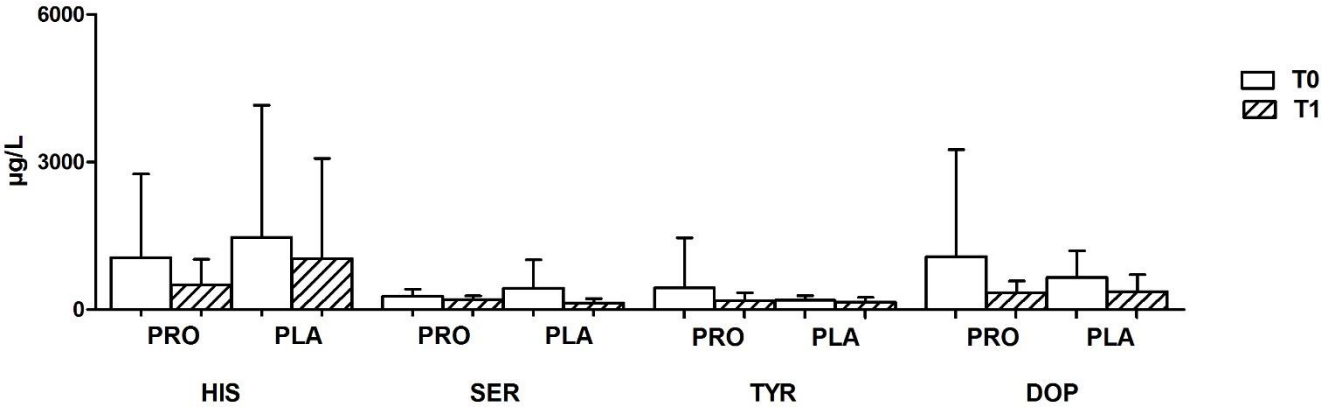

Figure 2. Histamine, Serotonin, Tyramine and Dopamine concentrations in urine samples of placebo and probiotic supplemented groups at T0 and T1. The results are expressed as mean values ± standard deviation (µg/L).

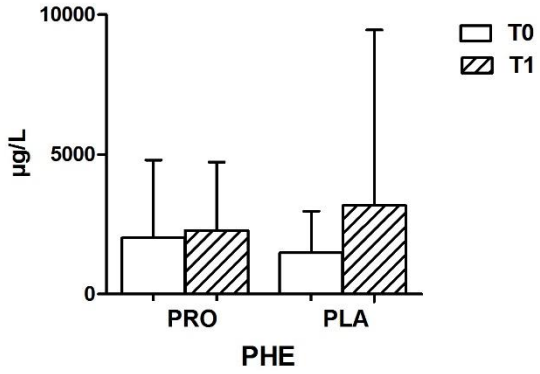

Figure 3. 2-phenylethylamine concentration in urine samples of placebo and probiotic supplemented groups at T0 and T1. The results are expressed as mean values ± standard deviation (µg/L).

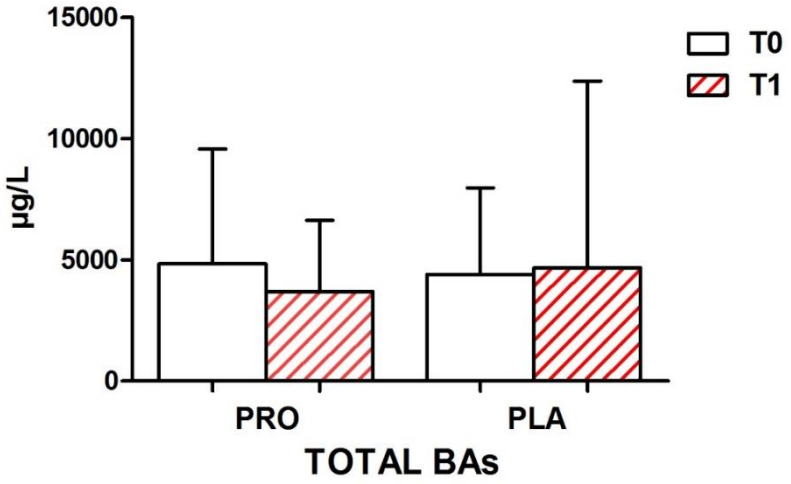

Figure 4. Total BAs concentration in urine samples of placebo and probiotic supplemented groups at and T1. The results are expressed as mean values ± standard deviation (µg/L).

6.5 DISCUSSION AND CONCLUSIONS

Ageing is a complex process that has adverse consequences on the structure and function of organs and systems in human body, leading to several age-related morbidities (Kregel and Zhang, 2007).

Since the recent discovery that BA in biological fluids and tissues can be markers of different and important diseases in clinical diagnosis medicine, we used BAs as biological indicators of health. In this study, we assessed the BAs levels in urine samples of elderly volunteers before and after probiotic supplementation (Gosetti et al., 2013).

Among BAs, polyamines (PAs) are an important group because their increase in blood or urine reflects the enhanced levels of PAs synthesis in rapid-growing cancer tissues/cells, since they are associated with decreased apoptosis, increased cell proliferation and expression of genes affecting tumour invasion and metastasis (Schipper et al., 2003; Thomas and Thomas, 2003; Bachrach, 2004; Soda, 2011). Maráková *et al*. (2020) demonstrated that Spd was significantly higher in patients with Crohn's disease respect to healthy subjects. In our study, the level of Put, Cad, Spd and Spm were monitored. In probiotic group, a reducing trend of Spd level was observed after supplementation, suggesting a beneficial effect of probiotics on GI health. On the contrary, in placebo group, Spd level remained stable.

Put and Spm levels decreased in both groups, suggesting a lack of specific effect of probiotic supplementation. It has been observed that a general reduction in polyamines, especially Put, in serum and urine of healthy human is physiological, since they are declining progressively with increasing age. It has been noticed also a reduced capacity of mammalian aged organs for PAs biosynthesis (Scalabrino and Ferioli, 1984). No specific effect has been shown on Cad, whose levels increased in probiotic group and decreased in placebo one, in both cases the variation was not significant. The study performed by Lyon *et al.* (1983) showed that Cad is toxic at very high doses, and it influences the toxicity of histamine. Increased levels have been also associated to genetic form of colon cancer (Pugin et al., 2017).

Also, the determination of other BAs, like catecholamines (CA), is important for clinical diagnosis. CAs are natural endogenous molecules involved in the regulation of psychomotor activity, emotional processes, learning, sleep and memory (Thomas and Thomas, 2003).

Balanced levels of Tyr, DA and 5-HT are important, with octopamine, for proper locomotion (Civelli et al., 1993). In our study, Tyr levels decreased after probiotic supplementation, while they remained unchanged in placebo group. Literature reports lower concentrations of Tyr in healthy volunteers, hypothesizing a beneficial influence of probiotic supplementation (Maráková et al., 2020). 5-HT and DA were detected in few samples (about 90% and 60% are null, respectively), probably due to their age-related reduction, as reported in several studies (Machado et al., 1986; Rauschenbach et al., 2011). In our case, DA concentration decreased in probiotic and placebo groups; on the

other hand, 5-HT levels remained mainly stable after probiotic supplementation and decreased in the placebo group. Since many elderly develop 5-HT deficiency due to age-related healthy and brain changes (dementia), poor diet, chronic stress, lack of sun exposure or insufficient physical exercise, the maintenance of baseline levels in urine is a positive result (Jenkins et al., 2016).

Together with 5-HT, Pea is involved in the regulation of mood and psychiatric conditions. Although not directly connected with the supplementation, in our study Pea levels increased in probiotic and placebo groups, suggesting a potential good psychological health status of the volunteers. An increase in Pea could be also caused by a unbalanced protein intake with diet (MacDonald et al., 2020).

High amount of His is associated with inflammatory bowel diseases, in local immune reactions and related inflammatory responses (Nieto-Alamilla et al., 2016; Maráková et al., 2020;). In our study, His levels decreased in both groups, a positive trend considering the inflammatory condition that characterizes ageing.

Finally, Trip and E were not detected in any urine samples of volunteers. Tryp levels could be very low in urine or absent, as reported by Maráková *et al.* (2020). Similar for E, since current evidence shows that adrenal medullary secretion and release of E are lower in older people (Esler et al., 2002).

Urine test proved to be inexpensive, quick, non-invasive and useful for clinical medicine (Bacaloni et al., 2013). Moreover, urine is nowadays the main source of BAs available, thanks to their longer biological half-life in comparison with plasma samples. Overall, the effect of probiotic supplementation was observed as a slight trend influencing some BAs more than others. However, the results remain inconclusive, due to detecting limitations and high biological variability. This highlights the need for more studies to investigate the potential mechanisms by which probiotics may influence BAs, and consequently healthy ageing.

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CHAPTER VII

EFFECTS OF PROBIOTIC SUPPLEMENTATION ON INFLAMMATORY MARKERS IN THE ELDERLY

7.1 ABSTRACT

Ageing is a complex phenomenon where inflammation plays a key role. In elderly, this low-grade, chronic inflammatory state is associated with a dysregulation of the immune system, leading to an increase in the concentration of pro-inflammatory cytokines. Most of the large, current epidemiologic studies of older adults have included tumour necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 (IL-1) as markers of inflammageing and age-related diseases and disability. Another potential marker of longevity genes on ageing and life span, acting in the mediation of these factors, seems to be Insulin-like growth factor 1 (IGF-1). Several studies revealed an inverse relationship between increased levels of cytokines and inflammatory markers and muscle strength/power in elderly. In our study, we aimed to determine the gene expression pattern, at the transcript and the protein level, of TNF-α and IGF-1 in an elderly community supplemented with probiotics-based diet. Firstly, our results showed a significant increase in circulating levels of IGF-1 in probiotic group, respect to placebo-supplemented one, in contrast with the expected reduction in elderly subjects, suggesting an IGF-1 modulation by GUT microbiota. As second findings, we evaluated TNF-α levels and its gene expression that resulted partially influenced by the dietary intervention. A significant increase was reported in mRNA expression of TNF-α in probiotic-supplemented group respect to placebo group. Moreover, the concentration of TNF-α decreased in both groups, even if without significance, suggesting that the probiotic supplementation didn't directly affect this parameter. In conclusion, the data analysed indicate that the probiotic supplementation used can modulate the GH/IGF-axis with positive effects on the pathogenesis of sarcopenia and other age-related morbidities. Also, the gene expression of TNF-α seems to be modulated by the supplementation, although no direct effect on the reduction of pro-inflammatory cytokines was observed.

7.2 INTRODUCTION

Ageing is characterized by physiological decline of biological functions. In humans, the ageing phenotype is extremely heterogeneous and influenced by the interaction of environmental, genetic and epigenetic alterations accumulated throughout the lifetime (Khan et al., 2017). In the complex process of ageing, the key role of inflammation has been clearly established in several epidemiologic studies. In older adults, this low-grade, chronic systemic inflammatory state is accompanied by dysregulation of the immune system, leading to an increase in the concentration of pro-inflammatory cytokines (Calder et al., 2017; Rea et al., 2018; Hutchinson et al., 2020;).

Among all the components of inflammageing, the most used in clinical practice as markers of age-related diseases and disability are: tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Singh and Newman, 2011; Baylis et al., 2013; Chung et al., 2019).

The molecular pathways involved in inflammageing are continuously object of study; another potential marker seems to be Insulin-like growth factor 1 (IGF-1), that is involved in mediating the effects of longevity genes on ageing and life span (Bartke et al., 2003).

IGF-1, also called somatomedin C, is a hormone similar in molecular structure to insulin. It is produced mainly in the liver and plays an important role in the first decades of life in the regulation of normal development and growth (Burgers et al., 2011). It acts as key regulator of cell proliferation and as inhibitor of cell apoptosis and necrosis (Ohlsson et al., 2009). In adults, IGF-1 has also important anabolic actions on the skeletal muscular system with stimulation of protein synthesis, improvement of the glycaemic profile and stimulation of production of nitric oxide with a positive effect on the endothelial functions of different districts. A recent topic of research has been the relationship between ageing and modification of activity of the growth hormone (GH)-IGF-1 axis (Vitale et al., 2019).

With ageing, the reduced biological activity of the GH-IGF-1 axis and the reduced secretion of GH (and IGF-1) occur gradually in both sexes throughout life, until only low levels can be detected in individuals aged ≥60 years (Krysiak et al., 2009; Vitale et al., 2019).

This phenomenon is called “somatopause” (Krysiak et al., 2009). Somatopause is associated to changes in body composition, metabolism, bone structure, physical performance, cardiovascular system functions, and increased morbidity and mortality (Krysiak et al., 2009; Vitale et al., 2019).

A similar profile is observed in adults with GH deficiency, who are affected by a reduction of bone and muscle mass and strength, an increased fat mass, dyslipidaemia, arterial hypertension, and cognitive decline (Vitale et al., 2019). Numerous observational studies documented a relationship between low IGF-1 levels and sarcopenia, frailty, cognitive changes and metabolic alterations such as metabolic syndrome and diabetes (Sonntag et al., 1999; Barbieri et al., 2003; Ferrucci and Fabbri, 2018). These studies, conducted on elderly subjects, have also suggested for IGF-1 a role as nutritional marker and its involvement in the development and progression of cardiovascular diseases (atherosclerosis, ischemic heart disease, stroke). In details, low IGF-1 levels have been associated with the development of ischemic heart disease and stroke while other population studies showed an association between high IGF-1 levels, but still within the normal range, and an increased risk of neoplasms. Since IGF-1 system has several pleiotropic effects on ageing phenomena, it has been studied in both animals and humans, proposing it as one of the potential longevity markers, despite some conflicting observations (Junnila et al., 2013). Experimental studies conducted on nematodes and mice have shown that the reduction of GH-IGF axis activity seems associated with longevity, but data obtained from human studies didn’t confirm (Vitale et al., 2019).

In elderly subjects, decreased as well as increased IGF-1 levels have been associated with a reduced life expectancy suggesting a U-shaped relationship between IGF-1 and mortality (Burgers et al., 2011). Moreover, some studies have shown an association between low levels of IGF-1 and an increased risk of cardiovascular disease while others a positive relationship between IGF-1 and cancer risk. So, it seems to exist an optimal set-point between the modifications of the biological activity of the axis GH-IGF-1 and longevity (Maggio et al., 2013).

Another aspect of ageing is the increased inflammatory activity in the blood, including higher circulating levels of TNF-α and other pro-inflammatory cytokines. Our interest was mainly focused on the TNF-α level in blood since it is another major player in the immune response as pro-inflammatory cytokine. TNF-α level increases with age and is associated

with age-related diseases; this pro-inflammatory mediator can also have a beneficial effect when it acts locally in the tissues, but it can be highly harmful when released systemically.

Clinical studies reported increased circulating TNF-α levels with ageing, suggesting an association with age-related degenerative pathologies; high plasmatic levels were found in type 2 diabetes mellitus and were associated with lower muscle mass and strength in older people (Lechleitner et al., 2002).

With increasing evidence of an association between TNF-α levels and age-related diseases, research has focused on the use of TNF-α inhibitors as drugs for the treatment and prevention of cardiovascular damage and Alzheimer's disease (Rea et al., 2018). Alzheimer's disease represents one of the most serious and severe health problems for elderly, because it is the main cause of dementia worldwide. There are many cytokines involved in the modulation of this kind of neuroinflammation, but TNF-α seems to play a crucial role in adults, both at the central and peripheral level. In this regard, many data show a positive correlation between TNF-α and Alzheimer's in both animal and human models (Pisa et al., 2017; Silva et al., 2019).

On the other hand, less known is the involvement of TNF-α in the occurrence of neoplastic phenomena; recent data seem to suggest the presence of an expression pattern of this cytokine associated specifically with the stage of tumorigenesis.

However, scientific literature shows quite clearly that TNF-α is a pleiotropic cytokine capable to modulate multiple homeostatic mechanisms, including the balance of the intestinal microbiota in the elderly population (Atzeni and Sarzi-Puttini, 2013; Nagpal et al., 2018). Indeed, TNF-α is considered the "master regulator" of inflammageing, that determines the onset of mitochondrial dysfunction, resulting in oxidative stress and alteration of the intestinal microbiota (Calder et al., 2017).

This chronic inflammatory state, through an alteration in the balance between the processes of synthesis and protein catabolism, is associated with reduced muscle mass and strength (sarcopenia), reduced plasma concentrations of IGF-1 and it is believed to potentially trigger or promote the development of major age-related diseases (Degens, 2010).

In addition to the other functions, IGF-1 is also involved in the modulation of inflammatory response and could therefore be a key element in the systemic effects of

the intestinal microbiota. During ageing, in fact, the alterations of microbial homeostasis and intestinal permeability involve a translocation in the blood stream of microbial products. These products, in particular lipopolysaccharides (LPS), promote a systemic inflammation that is potentially responsible for muscle atrophy and consequent deterioration of the quality of life of the elderly subject.

According to the presence of an inverse relationship between inflammation, seen as an increase in cytokines (and inflammatory markers), and muscle strength/power, we aimed to assess the balance between the inflammatory state and anti-inflammatory factors by determining the gene expression pattern, at the transcript and the protein level, of the two main markers involved in this process (TNF-α and IGF-1) in an elderly population supplemented with probiotic functional foods.

This part of PROBIOSENIOR Project was in line with both primary and secondary objectives of the study that were focused on the evaluation of the effects of a probiotic supplementation on the reduction of low-grade inflammation in elderly, from the evaluation of GUT microbiota and immunological status to other aspects of general health status.

7.3 MATERIALS AND METHODS

7.3.1 Quantification of mRNA and protein expression by qPCR and ELISA

7.3.1.1 RNA Extraction from White Blood Cells

The blood samples of elderly subjects enrolled were collected by the nursing staff involved in the project. After collection, the blood samples were immediately frozen and properly transferred to the laboratory of URDIS of 'University of Camerino', in San Benedetto del Tronto (Italy) in order to be processed and analysed, in collaboration with the team of Prof. Francesco A. Palermo.

Peripheral blood samples were distributed into tubes containing EDTA. The blood collected (3 ml) was diluted (1:1 v/v) with phosphate buffer salt (PBS) and mononucleate cells (PBMC) were isolated by centrifugation in gradient density, using Histopaque-1077 (1,077±0,001 g/ml; Sigma-Aldrich) according to the indications of the manufacturer. The fraction containing the PBMCs was transferred into a sterile test tube containing 1 ml of Trizol™ LS Reagent (Invitrogen Life Technologies, Milan, Italy) and the total RNA was

extracted following the protocol provided by the company (Thermo Fisher Scientific). The quantity and purity of RNA were assessed spectrophotometrically at absorbance of 260/280 nm.

7.3.1.2 Reverse Transcriptase-Polymerase Chain Reaction

The total RNA was treated with DNase (2U, 30 min at 37°C; Ambion) to eliminate contamination by genomic DNA. The effectiveness of treatment with DNase was then verified by performing a PCR in the which RNA treated with DNase was used as template. RNA extracted was reverse transcribed into cDNA using 5X All-in-One RT MasterMix kit, according to manufacturer's instruction (Invitrogen Life Technologies, Milan, Italy). The resulting cDNA was stored at -20°C until processing.

7.3.1.3 Real Time PCR analysis

For molecular analysis, a quantitative PCR based on the detection of amplified with the SYBR green method using the ABI 7300 (Applied Biosystems) system was performed. Specific primers were used for the amplification of target genes (Table 1). The expression value was normalized comparing with that of a constitutively expressed gene (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH). For the reaction was mixed 10 µl of BlasTaq^{TM} 2X qPCR MasterMix, 0.5 µl of FW primer (10 µm), 0.5 µl of RV primer (10 µm), 2 µl of cDNA and 7 µl of H_2O-DEPC. After the enzyme activation at 95°C for 3 minutes, the following cyclic thermo-profile was performed: denaturation 95°C for 15"; annealing-extension 60°C for 60" TNF-α and GAPDH, 61°C for 60" for IGF-1. The analysis of the dissociation curve (Tm), carried out by monitoring the signal of fluorescence during the increase of temperature from 60°C to 95°C for 15", showed that a single amplified containing the region of interest was generated. The results were calculated using the cycle threshold comparison method ($\Delta Ct = Ct_{gene\ target} - Ct_{GAPDH}$), with CtTG as the threshold cycle of the target gene and CtRG as the threshold cycle of the reference gene.

Table 2. List of Primers used in the study.

Gene	Primer sequence (5'-3')	Product (bp)	Reference
TNF-α	GTCAACCTCCTCTCTGCCATC CAAAGTAGACCTGCCCAGA	188	(Tamtaji et al., 2017)
IGF-1	CATGTCCTCCTCGCATCTCTA GCAGCACTCATCCACGATA	212	(Zhang et al., 2018)
GAPDH	AAGCTCATTTCCTGGTATGACAA CGTCTTCCTCTTGTGCTCTTGCTGG	126	(Tamtaji et al., 2017)

7.3.1.4 ELISA analysis

For the measurement of plasma levels of TNF-α and IGF-1, Human TNF-α and Human IGF-1 ELISA Kits were used, following the protocols provided by the manufacturer (MyBiosource In., San Diego, CA, USA). The absorbance (ABS) of each well was measured at 450 nm using a microplate reader (Biochrom^{TM}).

7.3.1.5 Statistical analysis

Data were analysed by One-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test, in order to evaluate the differences between the supplementations used at different time points. Statistical significance was considered when the probability value $P<0.05$.

7.4 RESULTS

7.4.1 TNF-α and IGF-1 levels at baseline

The molecular analysis of circulating factors and gene expression was carried out on 97 blood samples before (T0) and after (T1) the probiotic and placebo dietary intervention. Figures 1, 2 and 3 show respectively the results of mRNA expression of TNF-α, normalized for the housekeeping gene GAPDH, TNF-α and IGF-1 plasma concentration (expressed in pg/ml) at baseline conditions. The results at T0 were analysed and represented considering the residence structure and the gender, with the aim to appreciate the any variations and differences between them.

The TNF-α expression was characterized by a high variability between genders, showing an increased expression level in females respect to males. In addition, a

statistically significant difference was evidenced analysing the residence structure of volunteers; higher levels were detected in people living in boarding home than in private houses (Figure 1).

Plasma circulating levels of TNF-α seem to be not influenced by gender, showing in females and males a very similar concentration. On the contrary, as confirmed by its expression in Figure 1, TNF-α levels were significantly different according to the living place, showing higher values in volunteers in boarding homes than in private houses (Figure 2).

Analysing plasma levels of IGF-1, similar basal concentrations were observed in females and males. In line with the results reported for TNF-α, IGF-1 concentration increased referring to people living in boarding homes (Figure 3).

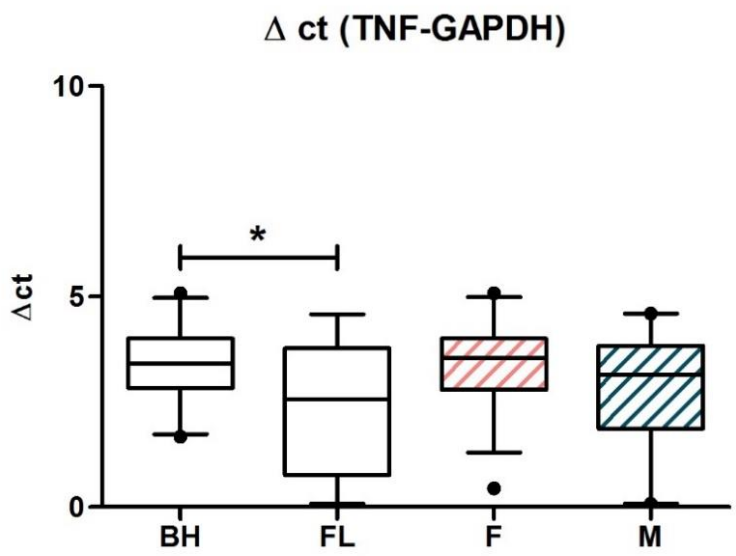

Figure 1. Box and whiskers plot (P5-P95) of mRNA expression of TNF-α at T0 (n=97). BH: boarding home subjects; FL: free-living subjects. F: females; M: males. **Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.*

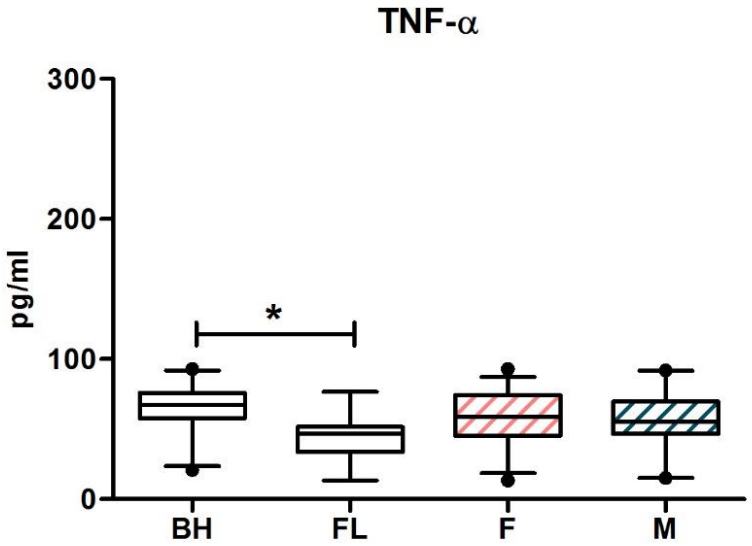

Figure 2. Box and whiskers plot (P5-P95) of plasma levels of TNF-α (pg/mL) at T0 (n=97). BH: boarding home subjects; FL: free-living subjects. F: females; M: males. **Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.*

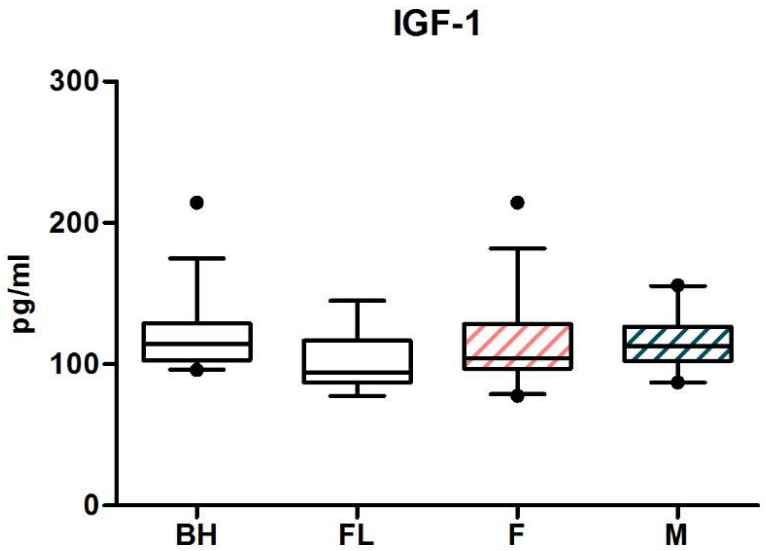

Figure 3. Box and whiskers plot (P5-P95) of plasma levels of IGF-1 (pg/mL) at T0 (n=97). BH: boarding home subjects; FL: free-living subjects. F: females; M: males.

7.4.2 TNF-α and IGF-1 levels after intervention

The molecular analysis of circulating factors and their gene expression was repeated at the end of dietary intervention, to evaluate the changes occurred after probiotic and placebo supplementation.

Figure 4 represents the mRNA expression of TNF-α in probiotic and placebo group. The results reported a significant increase of gene expression at T1 respect to T0, after probiotic supplementation. A slight, but nonsignificant, increment was observed also in the control group at T1 (Figure 4).

The levels of plasma circulating TNF-α at T1 are reported in Figure 5. It is possible to appreciate that the basal levels of TNF-α were comparable between the two groups analysed and that there was no significant difference in the protein concentrations after probiotic and placebo supplementation. A slight decrease in the mean values was observed in both groups (Figure 5). Different were the effects of the supplementation on IGF-1 levels at T1, shown in Figure 6. The results reported a statistically significant increase in IGF-1 concentration in probiotic group. The same trend was observed in placebo group, but without significant differences. In both groups, a higher intra-variability was observed at T1 compared with T0 (Figure 6).

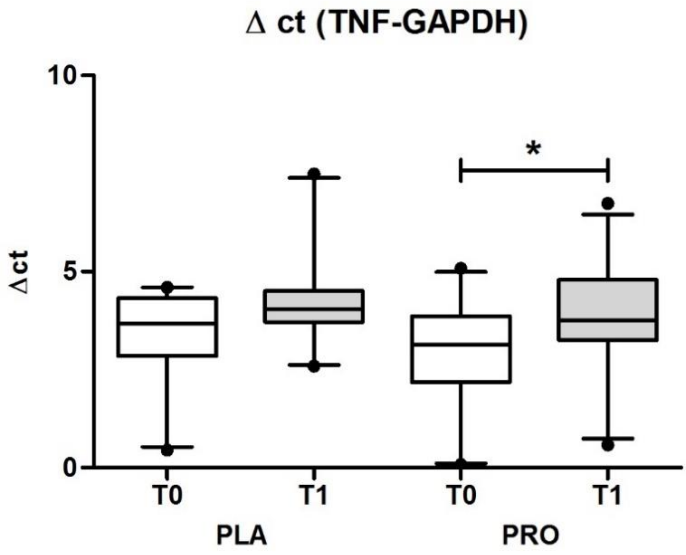

Figure 4. Box and whiskers plot (P5-P95) of mRNA expression of TNF-α at T0 and T1, in probiotic and placebo groups (n=97). **Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.*

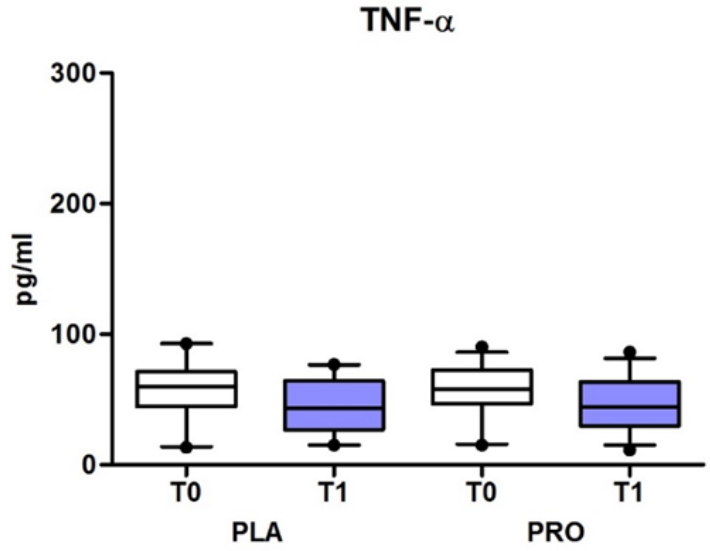

Figure 5. Box and whiskers plot (P5-P95) of plasma levels of TNF-α (pg/mL) at T0 and T1, in probiotic and placebo groups (n=97).

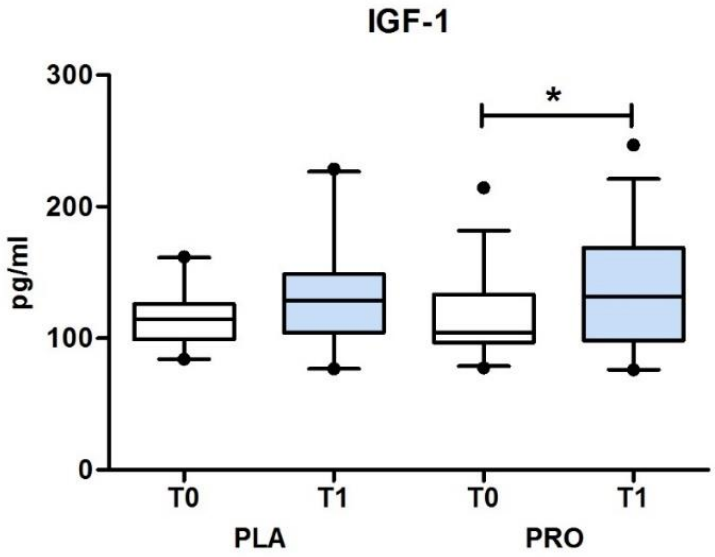

Figure 6. Box and whiskers plot (P5-P95) of plasma levels of IGF-1 (pg/mL) at T0 and T1, in probiotic and placebo groups (n=97). **Significantly different (P<0.05) from T0 by Tukey's test, One-way ANOVA.*

7.5 DISCUSSION AND CONCLUSIONS

Ageing is characterized by physiological decline of biological functions (Khan et al., 2017). In the complex process of ageing, low-grade inflammation plays a key role in the risk to develop age-related diseases and disabilities. In elderly people, an increase in the low-grade inflammatory markers associated with chronic conditions of ageing has been observed (Singh and Newman, 2011).

In this part of PROBIOSENIOR Project, that aimed to evaluate the effects of a probiotics-based diet on the low-grade inflammation in clinically healthy older people, we focused on the gene expression pattern, at the transcript and the protein level, of the two main markers involved in this process: TNF-α and IGF-1.

The results obtained at baseline showed that plasma levels of TNF-α were in line with those measured in clinically healthy subjects, with normal values of 75 +/- 15 pg/ml (Damas et al., 1989). Significant variations were observed between different residence structures, since higher TNF-α levels were detected in people living in boarding homes than private houses. The same trend was observed for mRNA expression of TNF-α, with statistically significant different values in boarding home and free-living subjects. This variability was expected by a heterogenic community with different habits, lifestyle, and health status. Indeed, several studies examined diet and lifestyle factors that could influence inflammation, oxidative stress, and the functional role of these immune/inflammation response genes, suggesting a strong correlation between them (Slattery et al., 2014a, 2014b).

On the contrary, circulating levels of IGF-1 at baseline showed a slight difference between people living in boarding homes and private houses, although without significance. Our results revealed that IGF-1, TNF- α and its gene expression were not influenced by the gender, as confirmed by literature. Studies hypothesize that gender-specific factors, such as hormones levels, may potential influence IGF-1 concentration, but it still needs further investigation (Ho et al., 2006). Current data suggest that the existing sex differences in IGF-1 levels, when present, are small in general healthy individuals (Aimaretti et al., 2008; Friedrich et al., 2010).

At the end of the dietary intervention, some interesting findings have been demonstrated.

Firstly, the study revealed a significant increase in circulating levels of IGF-1 in probiotic group, respect to placebo-supplemented one. These positive results are in contrast with literature, since in elderly, the hormones promoting muscle cells growth, such as growth hormone (GH) and insulin-like growth factor−1 (IGF-1), generally decrease (Brabant and Wallaschofski, 2007; Friedrich et al., 2010; Sattler, 2013).

Moreover, in sarcopenia and frailty conditions, increasing evidence suggests that the age-related low signalling of GH, IGF-1 and hormones are associated with the their

incidence and pathogenesis (Sorenson et al., 2008; Dai et al., 2010; Kim and Choi, 2013; Basualto-Alarcón et al., 2014; Cesari et al., 2017).

Recent studies revealed also the role of microbiota in the skeletal growth and homeostasis, describing a more complex interconnected system, the gut microbiota-skeletal muscle axis (Neish, 2009; Yan et al., 2016; Lahiri et al., 2019). The results obtained in our study agree with recent literature on the microbiota's ability to modulate circulating levels of IGF-1 in the host by increasing its synthesis (Yan et al., 2016; Yan and Charles, 2018). The increase of IGF-1 determines a stimulation of the somatotropic axis with very important effects both on the regulation of the "bone remodelling" and on muscle atrophy, two important aspects in elderly.

However, the specific mechanisms affecting IGF-1 variation and the pathways by which the microbiota modulates IGF-1 synthesis at systemic or local level are still poorly understood. The short-chain fatty acids (SCFAs) production by microbial metabolism may be one mechanism by which microbiota increases IGF-1 levels, both in the direct activation of the IGF-1 production at tissue level and in the indirect way mediated by the GH release at pituitary level (Yan et al., 2016). Another potential underlying mechanism of muscular system function changes is the age-related variation of biogenic amines levels (Rauschenbach et al., 2011; Seid and Traniello, 2005).

The second findings of this study were referred to TNF-α levels and its gene expression that resulted partially influenced by the dietary intervention. We observed a significant increase in mRNA expression of TNF-α in probiotic-supplemented group respect to placebo group, although a slight variation was noted.

Several researches demonstrate the ability of probiotics to modulate the immune system, but just few studies describe the mechanisms of action of these microorganisms, that were not completely established (Bermudez-Brito et al., 2012; Plaza-Díaz et al., 2017; Pistol et al., 2019; Plaza-Diaz et al., 2019). It has been also hypothesized the interaction between probiotics and cell signalling pathways related to inflammatory processes, suggesting the importance of strain specificity (Taranu et al., 2018).

Although clinical studies reported increased circulating TNF-α levels with ageing, and its correlation with age-related diseases, in our study the concentration of TNF-α decreased in both groups, even if in a not significant manner (Lechleitner et al., 2002).

The obtained results suggest that the probiotic supplementation didn't directly affect this parameter.

In conclusion, the data analysed would seem to indicate that the probiotic supplementation used is able to modulate the GH/IGF-axis with positive effects on the pathogenesis of sarcopenia and other age-related morbidities. In addition, also the gene expression of TNF-α seems to be influenced by the supplementation, although it did not have a direct effect on the reduction of pro-inflammatory cytokines.

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CHAPTER VIII

IMPACT OF PROBIOTIC SUPPLEMENTATION ON hsCRP CONCENTRATION AND HAEMATOLOGICAL PARAMETERS

8.1 ABSTRACT

Increasing evidence demonstrates that high sensitivity C-reactive protein (hsCRP) is not only an international recognized marker of low-grade chronic inflammation, but also an important risk factor associated with age-related diseases, including cardiovascular disease, hypertension, diabetes mellitus, and kidney disease. This study investigated the effects of 24-week probiotic functional foods-based diet on the reduction of plasma concentration of hsCRP in elderly subjects; general haematological parameters were also monitored for evaluating the physiological and pathological conditions. 97 elderly volunteers met inclusion criteria and were enrolled in the study. Blood samples were collected at baseline (T0) and at the end of the supplementation (T1) for hsCRP determination and haematological measurements. The main outcome of the study was the significant reduction of hsCRP levels in probiotic group, respect to placebo ($P<0.05$). Considering different supplementation time, the 6-month probiotic intervention resulted significantly more effective than the shorter one (<6 months). Finally, probiotic administration did not contribute to significant changes in blood parameters, although an improving in lipid profile was observed ($P>0.05$).

8.2 INTRODUCTION

Inflammageing plays a key role in vascular damage (CVD) and insulin resistance, increased risk of developing type 2 diabetes, cancer, sarcopenia, frailty, and neurodegeneration (Bruunsgaard et al., 2003; Payette et al., 2003; Vasan et al., 2003; Xu et al., 2003; Bruunsgaard, 2006; Trichopoulos et al., 2006; Tan et al., 2007; Wen et al., 2011; Beyer et al., 2012; Collerton et al., 2012; Jenny et al., 2012; Quaglia et al., 2014). Moreover, some research data suggest that low-grade inflammation has also predictive roles in mortality in elderly individuals affected by various pathologies, but other studies report conflicting opinions (Bruunsgaard et al., 2003; 2006; Jylhä et al., 2007; Alley et al., 2008; Wassel et al., 2010; Giovannini et al., 2011; Beleigoli et al., 2013; Ferrando-Martínez et al., 2013). Many studies investigate the use of various inflammatory biomarkers as predictors of health status in elderly subjects (Pepys and Hirschfield, 2003). Among the most commonly used indicators, high sensitivity C-reactive protein (hsCRP) is an internationally recognized marker of low-grade inflammation (Pearson et al., 2003). CRP levels increase in an age-dependent manner, due to proinflammatory cytokines that stimulate the synthesis (Ahmadi-Abhari et al., 2013). In addition, lower concentration was observed in healthy older adults than in people with age-related diseases or disability (Puzianowska-Kuźnicka et al., 2016). Besides age, many other factors can alter baseline CRP levels including gender, smoking status, weight, lipid levels, and blood pressure (Hage and Szalai, 2007). In the current literature, the most common diseases and conditions associated with increased CRP levels in older patients are: CVD and hypertension, diabetes mellitus, chronic kidney injury, neurodegenerative pathologies, sarcopenia and also mortality (Tracy et al., 1997; Cesari et al., 2003; Makita et al., 2005; de Rekeneire et al., 2006; Labonté et al., 2012; Song et al., 2014; 2015; Costello-White et al., 2015; Panickar and Jewell, 2015; Hosford-Donovan et al., 2016; ; Tegeler et al., 2016; Velissaris et al., 2017).

Indeed, in older adults without subclinical diseases at baseline, CRP was identified as a strong predictor of CVD (Cesari et al., 2003). In specific cases, it can be used as marker of advanced atherosclerosis and future acute risk of CV events (Li et al., 2007).

In elderly, increased CRP levels has been associated also with a reduction in physical functioning, subnormal levels of sex hormones and metabolic syndrome (Sattar et al., 2008; Kupelian et al., 2010; Fu et al., 2016; Sousa et al., 2016).

Another important role of CRP is as independent risk factor for impaired fasting glucose, impaired glucose tolerance and diabetes. Elevated haemoglobin A1c (HbA1c, %) and blood glucose levels are correlated with circulating levels of CRP in elderly (85 years old) (Wijsman et al., 2012).

Available evidence show that CRP is strongly associated with neurodegenerative diseases. In older adults affected by Parkinson's disease, CRP levels are significantly higher than in healthy subjects (Song et al., 2014; Akıl et al., 2015). Moreover, the risk of incidence of CVD in Parkinson's disease patients with high CRP levels is increased (Hassin-Baer et al., 2011). Another cross-sectional study reported that CRP plays a functional role in its pathogenesis and may be useful to assess Parkinson's disease severity (Zhang et al., 2011). Also Alzheimer's disease is influenced by elevated levels of CRP, leading to reduced cognitive function and survival time (Nilsson et al., 2011; Szewieczek et al., 2015). In these patients, also more severe dementia was observed (O'Bryant et al., 2010).

Although CRP is involved in several inflammatory processes and host responses to infection, it should not be used as the only predictor of CVD and other age-related pathologies in elderly people, because its action can vary in different clinical settings.

The measurement of blood parameters in elderly is a valuable and safe supporting instrument for evaluating the biological, physiological, and pathological conditions of subjects. Although the blood count is one of the most common and used tests for health assessment, it is difficult to define a reference population for laboratory assessment, because of the higher incidence of chronic morbidities in elderly (Zierk et al., 2020). Several studies examined the age-related physiological changes in clinical parameters and their association with obesity, hypertension, type 2 diabetes mellitus, kidney disease and other conditions.

It has been observed that ageing is associated with substantial changes in body composition, with a redistribution of a higher body fat and a reduced fat-free mass (Baumgartner et al., 1995; Beaufrère and Morio, 2000; Villareal et al., 2005).

Also the prevalence of hypertension increases linearly with age (Murakata et al., 2015). Murakata *et al*. (2015) observed in their study a close correlation of fasting plasma

glucose level or blood haemoglobin A_{1c} content with age. They evaluated the correlation referred to fasting plasma glucose level or for blood haemoglobin A_{1c} in longitudinal data for all the subjects under study. Also the lipid profile has been assessed, showing an increase in triglyceride and total cholesterol level with age. The gender seems to influence the variations of HDL- and LDL-cholesterol during ageing. However, the mechanisms responsible for the development of dyslipidaemia with age remain partially unclear.

Among the clinical parameters to monitor, the serum creatinine has been object of study to assess renal functionality. The age-related physiological changes result in an impairment of kidney function, increased creatinine level and development of CKD, whereas eGFR declines (Coresh et al., 2007; Yamagata et al., 2007; Weinstein and Anderson, 2010).

Our project aimed to evaluate the effects of 24-week probiotic functional foods-based diet on the reduction of plasma concentration of hsCRP in healthy elderly people. HsCRP was selected as primary target, because of its role as marker of inflammation and other age-related diseases mentioned above. HsCRP cut offs of less than 1 mg/l, 1-3 mg/l, and greater than 3 mg/l are commonly used for cardiovascular risk discrimination, especially among people with the metabolic syndrome, and incident diabetes (Musunuru et al., 2008). The levels of hsCRP in the blood are between 1 and 3 mg/l in the 70% of elderly population, and higher than 3 mg/l in the 30% (Imhof et al., 2003). Participants with hsCRP >1 mg/l were eligible and enrolled in our clinical study. Moreover, we measured general blood parameters to evaluate the health status of volunteers and record any changes after probiotic supplementation.

The principal claimed benefits of probiotics in elderly people are related to GUT environment, modulating the age-related gut microbiota imbalance, improving intestinal barrier function and gastrointestinal motility, and protecting against pathogens (Jenks et al., 2010; Hutchinson et al., 2021). In addition to all these effects, probiotics are largely studied because they have a role in either preventing or improving the outcomes of several health conditions, such as obesity, insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease, cancer, and immunodeficiency (Alipour et al., 2014; Sáez-Lara et al., 2016).

8.3 MATERIALS AND METHODS

8.3.1 Blood samples

Blood samples of 97 volunteers were collected into BD Vacutainer® tubes (Becton Dickinson, NJ, USA), were delivered to the Fioroni laboratory (San Benedetto del Tronto, AP, Italy) and analysed within 24 h. The samples were collected before the probiotic/placebo supplementation (T0) and within one day from the end of the treatment (T1). Blood samples were used for hsCRP determination by immunoturbidimetric method. An aliquot of the same samples was used for general haematological measurements. The blood tests performed determined: leukocytes, erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean haemoglobin (Hgb) concentration, mean corpuscular Hgb concentration (MCHC), red cell distribution width (RDW), platelets, neutrophils, eosinophils, basophiles, lymphocytes, monocytes, AST- and ALT- transaminases, lipid profile, glycaemia, blood urea, calcium, creatinine, sodium, potassium, calcium, albumin.

8.3.2 Statistical analysis

Data were analysed by One-way analysis of variance (ANOVA) (Tukey's Multiple Comparison Test), Mann-Whitney-Wilcoxon, and t-test. Statistical significance was considered when the probability value $P<0.05$.

8.4 RESULTS

8.4.1 HsCRP and haematological parameters determination

At baseline conditions, all enrolled subjects had a hsCRP level >1 mg/l. HsCRP distributions among men and women were very similar, without showing significant differences (data not reported). The effects of supplementation were observed analysing the difference of hsCRP value occurred between the end of supplementation (T1) and the starting time (T0), in probiotic and placebo group (Figure 1). In probiotic group, the hsCRP difference value was statistically significantly higher than that in the placebo group ($p<0.05$), showing the 6-month supplementation influenced on the low-grade inflammatory status of the subjects. Within the probiotic group, 33 out of the 103 enrolled subjects did not complete the experimental phase, taking the supplementation for three

months instead of six. Considering the two different periods of treatment, 6 month-supplementation showed a significant effect ($p<0.05$) on the protein level of the subjects, respect to those of a shorter one (<6 months), suggesting the important role of the supplementation time (Figure 1).

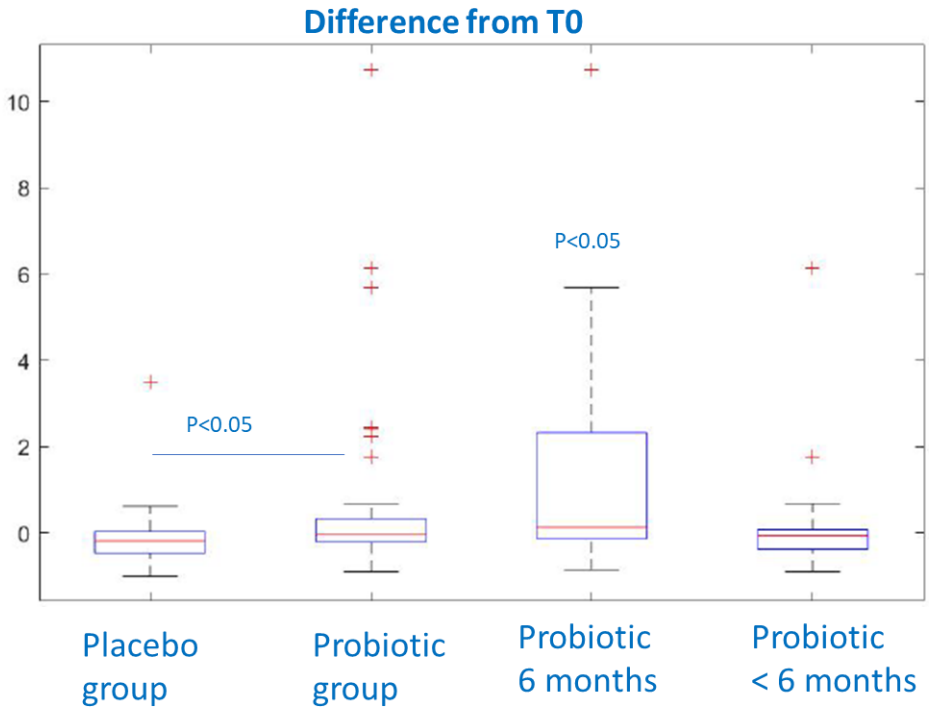

Figure 1. Difference values of hsCRP level between T0 and T1 related to the two experimental groups and times of probiotic supplementation.

The effect of probiotic dietary intervention on haematological parameters of the subjects are shown in Table 1 and Figure 2. Table 1 reports the mean values of all the blood parameters analysed in placebo and probiotic group, at two different time points (T0 and T1). There are no statistically significant differences in the levels between the two groups. However, despite a high inter-variability was observed in each group, all the parameters remained within the reference ranges.

Figure 2 compares the lipid profile at T0 and T1 of the two groups under study. Overall, no statistically significant differences between treatments were found for total cholesterol, HDL- and LDL-cholesterol, and triglycerides. However, a positive increasing trend was observed between T0 and T1 in the subjects receiving probiotic supplementation. On the contrary, the other parameters maintained the same levels of baseline, with just little variations.

Table 1. Blood parameters at T0 and T1 in placebo and probiotic group. Results expressed as means ± SD. P: significance levels between T0 *vs* T1 in placebo and probiotic group. NS: differences not statistically significant (n=97).

	PLACEBO (MEAN ± SD)		P	PROBIOTIC (MEAN ± SD)		P
	T0	T1		T0	T1	
Leukocytes (thousand x mm³)	6.45±1.76	6.46±1.66	NS	6.27±1.81	6.35±1.8	NS
Erythrocytes (million x mm³)	4.41±0.55	4.4±0.58	NS	4.37±0.61	4.28±0.66	NS
Hgb (gr/dl)	17.51±22.94	12.95±1.67	NS	12.83±1.7	12.46±1.75	NS
Haematocrit (%)	39.51±4.77	39.38±5.12	NS	38.71±4.86	37.82±5.09	NS
MCV (fl)	89.80±4.74	89.48±4.79	NS	88.93±4.61	88.66±5.14	NS
Mean Hgb concentration (picograms)	29.88±2.12	29.51±1.99	NS	29.46±1.95	29.26±2.03	NS
MCHC (gr/dl)	32.89±1.79	33±0.97	NS	33.13±1.14	33.3±2.41	NS
RDW (%)	17.20±10.21	13.6±0.91	NS	16.29±7.79	13.8±1.13	NS
Platelets (thousand/mm³)	204.26±50.72	204.37±38.69	NS	221.55±65.67	229.79±63.04	NS
Neutrophils (%)	58.54±7.97	72.8±86.65	NS	57.07±8.84	56.75±10.48	NS
Eosinophils (%)	3.83±2.21	3.32±1.93	NS	3.26±1.66	3.55±1.67	NS
Basophils (%)	0.74±0.35	0.69±0.32	NS	0.81±0.56	0.75±0.44	NS
Lymphocytes (%)	29.49±9.09	31.67±8.14	NS	29.96±9.04	30.13±9.14	NS
Monocytes (%)	9.42±4.86	8.32±1.88	NS	8.38±2.4	8.85±2.33	NS
AST-transaminase (Enzyme unit/L)	20.78±9.52	19.97±8.43	NS	19.81±7.17	20.55±6.22	NS
ALT-transaminase (Enzyme unit/L)	14.25±7.13	14.71±6.54	NS	17.68±9.8	19.84±15.81	NS
Cholesterol (mg/dl)	180.48±37.12	178.85±39.15	NS	179.64±42.95	174.55±37.51	NS
HDL cholesterol (mg/dl)	47.37±11.17	49.78±17.99	NS	49.74±11.09	55.97±48.13	NS
LDL cholesterol (mg/dl)	115.63±34.97	114±38.57	NS	112.67±36.79	109.6±34.03	NS
Triglycerides (mg/dl)	126.65±53.00	124±69.24	NS	130.59±64.02	127.21±58.78	NS
Glycaemia (mg/dl)	94.22±32.17	89.81±21.94	NS	98.67±45.95	97.69±36.84	NS
Blood urea (mg/dl)	42.34±17.39	46.19±19.38	NS	49.79±21.27	51.27±26.97	NS
Creatinine (mg/dl)	1.01±0.44	0.97±0.4	NS	1.03±0.31	1.02±0.36	NS
Sodium (mmol/L)	140.44±2.56	135.81±24.44	NS	139.83±2.46	140.24±2.1	NS
Potassium (mmol/L)	4.2±0.46	4.38±0.44	NS	4.34±0.39	4.54±0.41	NS
Calcium (mEq/L)	6.68±2.49	6.68±2.6	NS	7.55±2.39	7.42±2.47	NS
Albumin (g/dl)	8.94±26.79	3.79±0.33	NS	3.85±0.35	3.79±0.33	NS

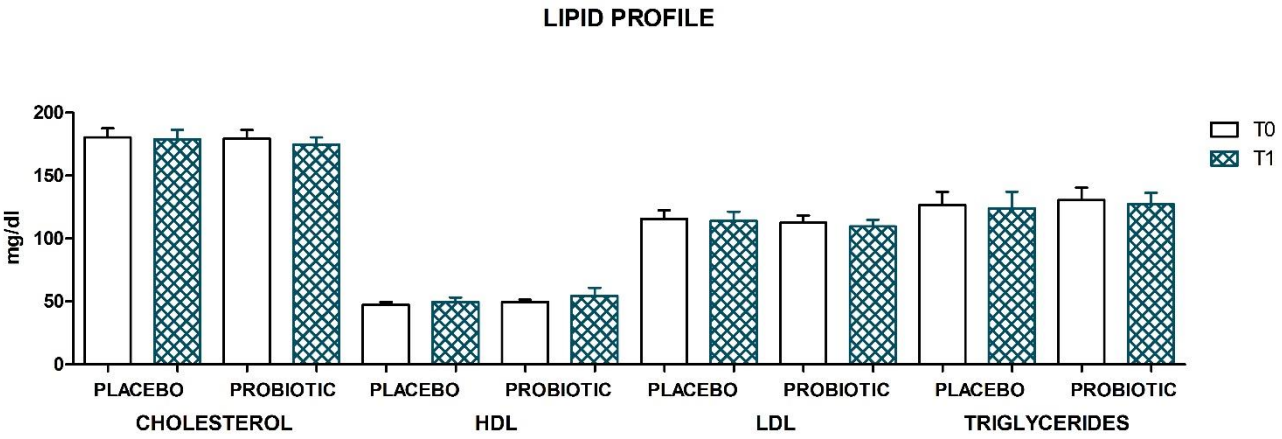

Figure 2. Total cholesterol, HDL- and LDL-cholesterol, and triglycerides concentrations in probiotic and placebo group, at T0 and T1. Results expressed as means ± SEM (n=97).

8.5 DISCUSSION AND CONCLUSIONS

Chronic inflammation is considered a key factor contributing to ageing, and evidence reveal that this pathophysiological process can be modulated. The physiology of ageing and mechanisms responsible for age-related diseases and disorders are continuously investigated. Therefore, the main objective of the present study was to investigate the effects of a probiotics-based diet on the low-grade inflammation in clinically healthy older people, by monitoring hsCRP levels before and after probiotic supplementation. HsCRP is an acute phase protein recognized as marker of low-grade chronic inflammation (Mazidi et al., 2017); its levels increase with age (Ahmadi-Abhari et al., 2013). HsCRP is also associated with cardiovascular risk, diabetes, and neurodegenerative pathology (Cesari et al., 2003; Pearson et al., 2003; de Rekeneire et al., 2006; Song et al., 2015). The results of our study confirmed that a significant proportion of healthy elderly people has hsCRP concentration between 1 and 3 mg/l or higher than 3 mg/l, as expected for this community and reported by several epidemiological studies (Imhof et al., 2003; Jiang et al., 2013). In this study the difference between hsCRP levels at T0 and T1 was significantly higher in probiotic group than in placebo one ($P<0.05$). A further subgroup analysis was performed, considering the supplementation time. It revealed that the six-month supplementation had significant effect ($P<0.05$) on hsCRP concentration respect to the shorter one (<6 months). These positive findings highlight that consumption of probiotic supplements results in a significant reduction in hsCRP levels, and a longer

supplementation of six months is required in elderly people. Mazidi *et al.* (2017), in a meta-analysis of randomized control trials, investigated the impact of probiotic administration on CRP concentration, confirming our results. Several mechanisms have been proposed to explain the effects of probiotics consumption on hsCRP. One is the modulation of GUT microbiota and the consequently production of short-chain fatty acids in the colon. This situation results in a decreased enzymatic synthesis of hepatic hsCRP. Another suggested mechanism is that probiotics influence increasing glutathione (GSH) levels, scavenging superoxide and hydroxyl radicals, and decreasing expression of interleukin-6 (IL-6) in adipocytes. The reduced expression of IL-6 results in low hsCRP levels (Hegazy and El-Bedewy, 2010; Asemi et al., 2013).

In the present study, also general haematological parameters were measured before and after probiotic/placebo supplementation. Overall, no significant changes were observed between T0 and T1, in probiotic and placebo group. All the recorded parameters were in line with the reference ranges. However, analysing the lipid profile, an interesting variation in HDL-cholesterol level was observed. HDL-cholesterol values increased, although not significantly, after probiotic supplementation, respect to placebo group. Total cholesterol, LDL-cholesterol and triglycerides levels did not show evident variations. Several studies reported the improving effects of probiotics administration on blood lipids (Salahuddin et al., 2013; Costabile et al., 2017).

We can conclude that probiotics administration has a significant effect on the reduction of hsCRP in elderly people. On the other hand, the supplementation did not significantly affect haematological parameters, except for lipid profile that was slightly improved.

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CHAPTER IX

INTERACTIVE NUTRITIONAL EDUCATION INTERVENTIONS FOR ELDERLY PEOPLE TO PROMOTE HEALTHFUL AGEING

9.1 ABSTRACT

The higher incidence of malnutrition and age-related diseases and disabilities is becoming a severe phenomenon. Among multiple responsible factors, the daily diet has a key role in the prevention and modulation of health conditions in elderly. Since the difficulties to manage the nutrition according to the individual energy requirements and physio-pathological status, it is crucial to develop specific programs addressed to elderly people, caregivers and families. Practical education activity on proper nutrition represents the best strategy to improve healthful ageing, preventing malnutrition and relative morbidities. In this pilot project, we developed an activity to raise people's awareness about nutrition and the use of probiotic supplements, supporting and improving healthful behaviours. This program involved the elderly community of two different boarding homes, and the caregivers. The method provided five videos on basic nutrition topics, and final questionnaires to collect feedback about its impact and effectiveness on audience. This study demonstrated that this innovative communication method is a valid alternative to the live teaching approach for elderly. Moreover, most people recognized a positive impact on their daily nutritional habits. In conclusion, this intervention certainly contributed to improve the knowledge on nutrition and probiotics, in both elderly and caregivers, also providing a basis for designing effective nutritional education programs for older adults.

9.2 INTRODUCTION

The continuous increase in the aged population is leading to several age-related diseases and higher medical costs (Kim et al., 2012). A multidisciplinary approach is necessary to counteract this phenomenon. Adequate nutrition is essential to health and quality of life, especially for older adults (Xu et al., 2015; Pelclová et al., 2018). Improvements in nutrition can prevent, modulate or ameliorate many conditions typical of elderly, such as malnutrition (Clegg and Williams, 2018). Malnutrition is very common because daily food consumption tends to change with old age, due to the difficulties to manage the nutrition according to individual energy requirements.

Therefore, it becomes crucial to educate people about proper nutrition to understand how a balanced diet influences their health-related quality of life. Nutrition is a key factor in successful ageing as food is not only essential to physiological well-being but also contributes to social, cultural, and psychological quality of life. In the modern societies, a shift is ongoing to emphasize prevention, and nutritional education is crucial for all age groups. Particularly, several studies have described the positive effect of education activities or counseling for the elderly (Yoon and Lee, 2006; Choi et al., 2007; Park et al., 2007; Yim, 2008; Kang et al., 2009).

While attention to nutrition has been increasing, overall recognition of the importance of nutrition for healthy ageing developing specific educational programs are still important aspects to improve.

Prevention and education programs should focus on simple recommendations for a healthy, varied and balanced diet able to guarantee basic nutrients. Since dietary guidelines can be confusing and complicated to understand, practical and realistic approaches are a better strategy to optimize learning and future daily applications of knowledges by the elderly.

Moreover, an effective program should not be focused only on the individual level, but it should also involve the social and environmental levels, involving caregivers and families. A community-based activity can simultaneously improve socialization and the accessibility to nutritional knowledges. Dietitians and other nutrition educators play a large role in the development and implementation of this kind of interventions.

This study was designed to evaluate the effects of nutritional education on elderly

subjects living in two selected boarding homes. This is a pilot activity developed in parallel with PROBIOSENIOR project, with the purpose to use these preliminary data for establishing guidelines for nutritional management programs specifically targeted to older adults.

9.3 MATERIALS AND METHODS

9.3.1 Nutritional education activities: video-pills

The nutritional education intervention was implemented in the boarding homes "A. Chierichetti" in Gagliole (MC) and “ASP Lazzarelli” in San Severino Marche (MC). The activity was offered to the elderly subjects hosted in the structures, who joined PROBIOSENIOR project. The aim of this activity was to raise people’s awareness about nutrition, supporting and improving healthful behaviours. Unfortunately, this project was realized during the health emergency of Covid-19 with the impossibility to access the boarding homes. Consequently, to continue with this activity, five videos were prepared and shown to the elderly subjects by the caregivers, using tablets devices. The language used was simple and easy to understand by the auditors. These “video-pills”, recorded in Italian, were focused on basic topics, including nutrition in old age, importance of hydration, “Healthy Eating Plate”, five colours of health, and probiotics and prebiotics, considering their central role in PROBIOSENIOR project. At the end of each video practical solutions were proposed to create a sort of interaction with the subjects and make the activity more interesting.

9.3.1.1 Nutrition for older adults

The first video, composed by a series of slides with comment, (Annex 6; Slides 1- 10) is a general introduction about the main changes that occur in elderly organism (Figure 1), including the reduction of lean mass, the increasing percentage in the fat mass, the alteration in the perception of senses and stimuli, the chewing difficulties and the alteration of gastrointestinal system’s functionality and equilibrium (Slide 4).

One of the main goals in elderly population is to avoid malnutrition, both in excess and in defect, because it could have bad consequences (Slide 4). In this study, we also monitored the nutritional status using the Mini Nutritional Assessment questionnaire, at baseline and at the end of the supplementation.

A healthy, complete and balanced diet is fundamental to counteract these conditions, and the Mediterranean diet seems to be a good nutritional strategy to promote healthy ageing (Roman et al., 2008; Mazza et al., 2021). The adherence to the Mediterranean diet demonstrated beneficial effects to prevent several pathological conditions, from metabolic to cognitive disorders. In addition, positive effects on organs and systems, like muscular and cardiovascular systems, have been reported (Mazza et al., 2021) (Slide 5).

This nutritional style is based on high quality, seasonal, and various foods with well-balanced macro and micronutrients.

Firstly, cereals, legumes, vegetables, and fruits must represent the main source of energy of the daily diet. These foods are rich in carbohydrates, mostly starch and fibre, vitamins, mineral salts, antioxidants molecules and even a percentage of proteins (Slide 6).

In absence of specific pathologies, a proper intake lipid (30-35%) is also important, according to the energy requirements of subjects. Lipids are important in the diet as sources of energy, essential fatty acids (Omega-6 linoleic acid and Omega-3 linolenic acid), and fat-soluble vitamins (A-D-E-K) (Slide 7). They are fundamental components of the cell membrane and the main actors in the production of several molecules useful to our organism, likes hormones. Moreover, they contribute to satiety, diet's flavour, and palatability. It is strongly recommended to prefer extra-virgin olive oil (EVOO) as main dressing, respect to butter or other fats, rich in saturated fatty acids. EVOO contains unsaturated fatty acids and polyphenols which are linked to significant health beneficial effects (Nikou et al., 2019). On the other hand, some good practical strategies to monitor the intake, without exceeding, are eliminate the visible fat from meat and limit its consumption, limit fried foods and fatty cheeses, eat white and blue fish.

Finally, an optimal protein intake is recognised as a key dietary-related actor of healthy ageing (Slide 8). Proteins are essential to ensure the good maintenance of lean mass and muscle function, preventing sarcopenia and risk of falls (Donaldson et al., 2018). An adequate amount of high-quality proteins should be present at each meal, preferring fish, legumes, eggs, meat and soft cheese. It is recommended also to improve and more evenly distribute the daily intake of animal and plant-based proteins (Paddon-Jones et al., 2015).

A critical aspect is that elderly people are not a homogeneous group. Each subject has individual energy requirement and nutritional needs, according to the physio-pathological status. Hence, it is difficult to define a single model of diet that is valid for everyone. The best approach is to define simple guidelines and practical tips to be adopted as much as possible basing on individual abilities.

Another important aspect to consider is the environment where older adults live and eat. There are various factors that influence their eating behaviour and habits, from social relationship, food access, and proposed meals, to healthy nutrition information. The findings suggest that a positive social environment may be used to encourage healthy eating (Kvalsvik et al., 2021).

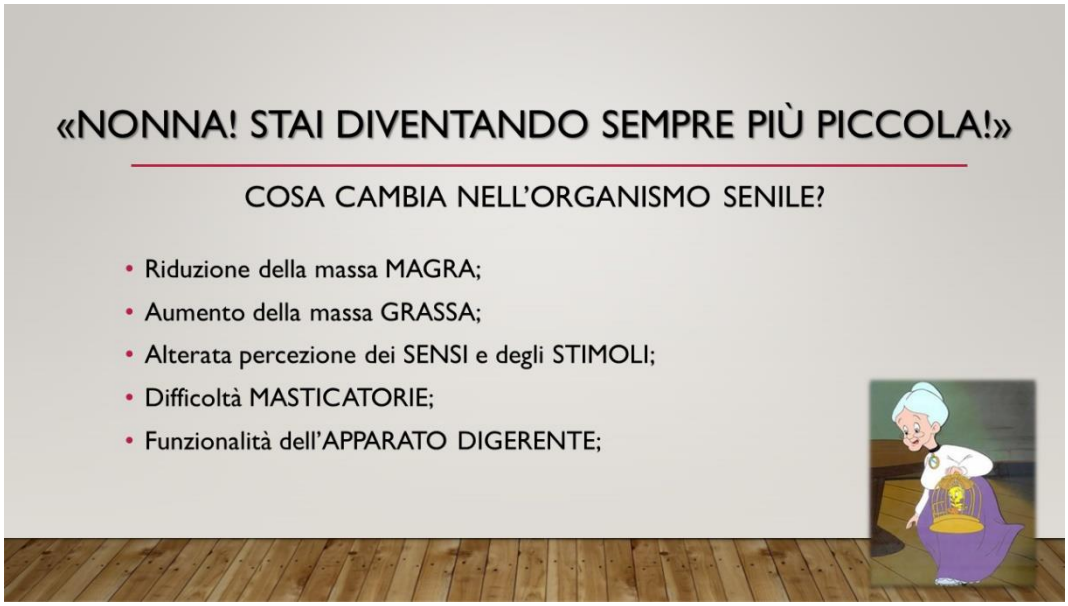

Figure 1. Example of slide from "Nutrition for older adults".

9.3.1.2 The importance of water

The second video (Annex 7; Slides 1-6) was developed to propose guidelines and practical tools to better manage hydration in elderly people, preventing dehydration.

Life has evolved in water, and it represents one of the most important nutrients, besides being the main constituent of our body (63%) (Figure 2). Water is essential for several biological functions, such as nutrients and wastes transport, temperature regulation, structures maintenance, and cell functions support (Ferry, 2005). Water maintains skin and mucous membranes, and it ensures the correct consistency of the

intestinal contents, contributing to a regular intestinal transit. It does not provide energy and calories (Slide 2).

The water requirement for an individual is defined as the quantity of water necessary to maintain homeostasis of both intra- and extracellular liquid compartments. The individual requirement changes in function of age, physical activity level, etc. Hence, recommended water intake for a population is calculated on the daily energy expenditure, about 0.25 mL/kJ (1 mL/kcal) for adults.

Another important aspect, especially in elderly people, is the maintenance of "water balance", the ratio between liquid intakes and liquid losses, to guarantee a good state of health in the short, medium and long term (Slide 3). The modifications in water metabolism and fluid imbalance that occur with ageing increase the risk of dehydration, especially in frailty subjects (Ferry, 2005). Dehydration is a serious risk factor of morbidity and mortality. It is responsible of the hospitalization of many patients, and it may be fatal. Common symptoms of dehydration are thirst and dry mouth/tongue (Shaheen et al., 2018). This is particularly true in the elderly, for whom the age-related physiological decline influences the thirst sensation, that occurs only in case of real significant water deficit (Kenney and Chiu, 2001). In addition, other clinical indicators of dehydration are variations in body weight, weak, fatigue, hypotension, concentrated urine, muscle cramps, headache and mental confusion. The symptoms are related to the state of dehydration, leading in the most severe cases to dried skin and mucous membranes, skin redness, loss of appetite, apathy, nausea and vomiting, tachycardia, and risk of coma. A persistent state of dehydration compromises both physical and mental capacities with severe consequences for the health. Moreover, it increases the risk of kidney, cardiovascular, gastrointestinal and urinary diseases (Picetti et al., 2017).

Elderly people are therefore particularly vulnerable, and it is fundamental to adopt strategies for preventing dehydration (Slides 4, 5).

Although the approach must be multidisciplinary, some practical tips to induce elderly people to drink enough are:

- Anticipate the sense of thirst by drinking about 1.5-2 L of water per day.
- Drink frequently, in small quantities, and slowly, especially if the water is cold. Drink throughout the day.

- Consider the wide offer of alternative beverages like tea, fruit juices, infusions, milk, and soup.
- Consume water-containing foods such as fresh vegetables, fruit, fresh cheese, and yogurt.

Finally, it is necessary to inform elderly people about the importance to drink enough, but also caregivers and health care professionals should be constantly aware of the risk factors and signs of dehydration in elderly patients.

Figure 2. Example of slide from "The importance of water".

9.3.1.3 The healthy eating plate: how prepare a balanced meal

"The healthy eating plate" is the main topic of the third nutritional education video (Annex 8, Slides 1-5). This concept has been proposed by Harvard University, in 2011. It was born to help people to combine proper amounts of high-quality foods, creating healthy and balanced meals, in line with Mediterranean diet (Figure 3; Slide 2). This simple guide consists in several indications and suggestions to apply every day, with the purpose to satisfy the nutritional requirements.

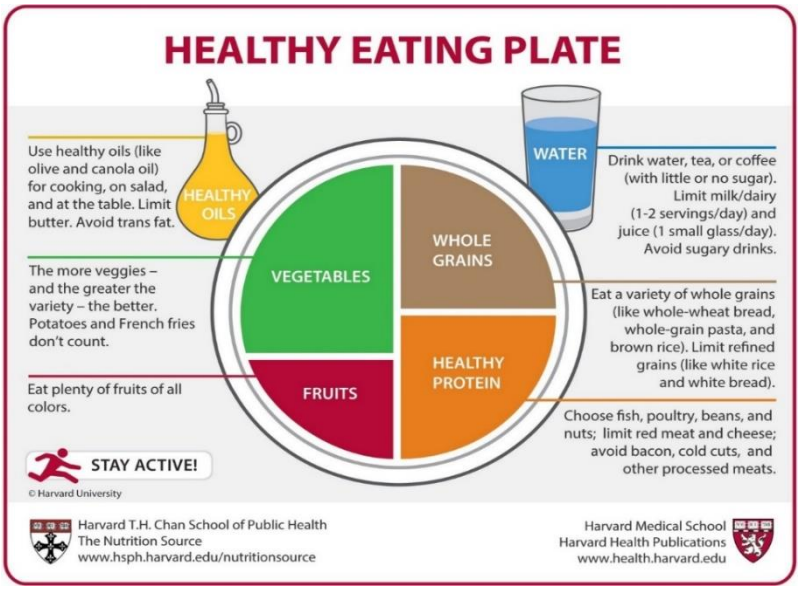

Figure 3. "The Healthy Eating Plate", Harvard University. Copyright © 2011.

With ageing, nutrition needs to change in accordance with physiological changes (altered functionality of some body systems), specific energy requirement, and lifestyle (less physical activity, different habits). In 2015, the Human Nutrition Research Centre on Aging (HNRCA) partnered with AARP Foundation in conjunction with the updated 2015-2020 Dietary Guidelines for Americans designed a practical guide specifically tailored for older adults: "MyPlate for Older Adults" (Figure 4).

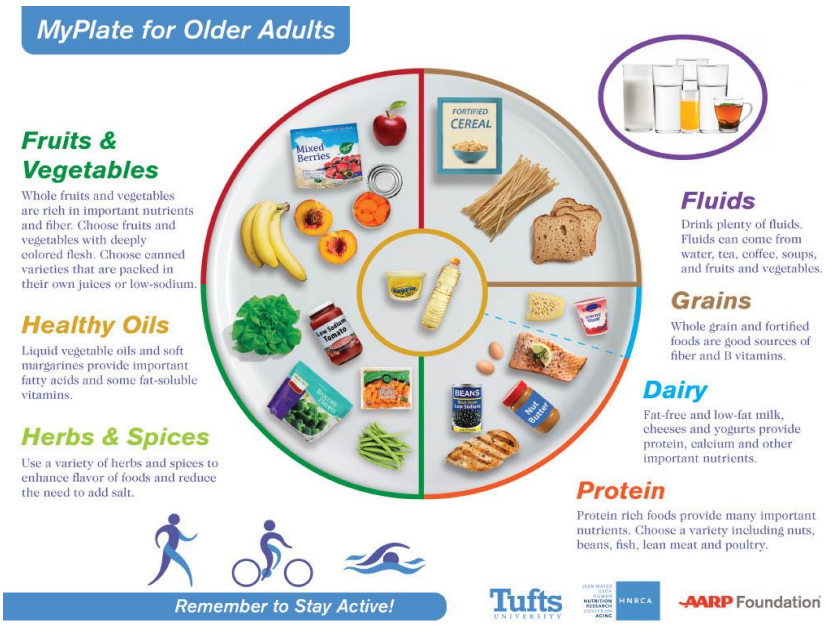

Figure 4. "MyPlate for older adults".

Some simple recommendations proposed in the video to apply for building a healthy and balanced diet are (Figure 5; Slides 3 and 4):

- Compose the main part of the meal with vegetables and fruit, filling about half of the plate. Vary your diet, preferring fresh and organic seasonal products.
- Choose whole grains for about ¼ of the plate. Vary frequently the carbohydrates sources, alternating pasta, rice, bread and other grains such as oats and spelled or even corn polenta if you like it!
- Add a portion of high-quality proteins in the last quarter of the plate, preferring white meat, white and blue fish, eggs and legume.

Limit the consumption of red and processed meats, such as bacon, sausages, and cold cuts to once a week. Limit the consumption of dairy products to a maximum of 2 times a week, choosing soft cheeses. Consume milk and natural white yogurt, to enrich with fresh fruits.

- Use extra virgin olive oil as main dressing. Limit animal fats such as butter and other vegetable oils. A right portion of extra virgin olive oil is around 10-12 grams per meal, about a tablespoon.
- Drink enough water. Among the alternative beverages, prefer tea and natural herbal teas respect to other drinks rich in sugars.
- Also, moderate physical exercise is suggested, especially in elderly, to maintain the body active and prevent the muscle loss.

As final activity, the auditors were invited to prepare together a healthy eating plate to show them that it is a simple thing to practice in their everyday life (Slide 5).

Figure 5. Example of slide from "The healthy eating plate".

9.3.1.4 The colours of health: fruits and vegetables

The fourth video is focused on the importance of a colourful diet (Annex 9; Slides 1-8). Fruits and vegetables are foods rich in vitamins, minerals, and bioactive compounds, like phytochemicals, carotenoids, anthocyanidins, and flavonoids (Oude Griep et al., 2011; Si and Liu, 2014). These essential nutrients are also rich in fibres and water, guarantying satiety and improving the hydration status (Slide 2). Hence, they have maintained a prominent place in the daily nutritional guidelines set by departments and ministries of health worldwide. Research has demonstrated that increasing fruit and vegetables consumption promotes healthy ageing, protecting against several chronic diseases by lowering oxidative stress and inflammation (Vincent et al., 2010; Nicklett and Kadell, 2013; Xie et al., 2013) (Figure 6). Fruits and vegetables are mainly classified into 5 colour groups: blue/purple, green, white, yellow/orange and red. The colour of the edible portion reflects the presence of pigmented phytochemicals, that can be considered an indicator of their nutrient profile and protective role (Oude Griep et al., 2011; Minich, 2019) (Slide 3).

- **Red:** watermelon, blood orange, beetroot, cherry, strawberry, tomato, radish, beetroot. They are rich in powerful antioxidants, effective in the prevention of

cardiovascular diseases and tumours by counteracting the production of free radicals. A role is recognized also on the urinary tract and on memory (Slide 4).

- **Yellow-Orange:** apricot, orange, carrot, clementine, kaki, lemon, mandarin, melon, medlar, pepper, peach, nectarine, grapefruit, pumpkin. They exert beneficial effects on eyesight, skin and immune system; endocrine-regulating activity and maintenance of gastrointestinal health; promotion of the well-being of our organs and systems, counteracting the production of free radicals (Slide 4).

- **Green:** asparagus, basil, chard, broccoli, artichoke, cabbage, cucumber, chicory, turnip greens, endive, kiwi, lettuce, parsley, rocket, spinach, grapes, zucchini. Important for sight, bone, teeth and skin health. An important action in the prevention of neurological and cardiovascular diseases (Slide 5).

- **Blue-Violet:** figs, berries, aubergines, plums, radicchio, black grapes. Beneficial effects on blood circulation; protective role of the urinary tract, memory and in the defence against free radicals (Slide 5).

- **White:** garlic, cauliflower, onion, fennel, mushrooms, apple, pear, leeks, celery, walnuts, hazelnuts, almonds, chestnuts. Important role in the prevention of tumour formation by counteracting cellular aging; maintenance of blood cholesterol levels (Slide 6).

The recommended amount is at least 3 and 2 portions of fruit and vegetables per day. The reference portion for fruit is 150 grams, while for vegetables is 80-200 grams (Slide 7).

In conclusion, the consumption of fruits and vegetables has numerous benefits, especially in elderly people. Since their daily intake is often less than what is recommended by guidelines, it is important to develop effective nutritional strategies.

Figure 6. Example of slide from "The colours of health".

9.3.1.5 Probiotics and Prebiotics: allies of the health

The last video was focused on probiotics and prebiotics, considering the focal point of PROBIOSENIOR Project (Annex 10, Slides 1-10). The video briefly described the role of probiotics and prebiotics in human health and well-being.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", while the term prebiotic indicates "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Hill et al., 2014; Gibson et al., 2017;) (Slide 4). They can be used as non-invasive supporting treatments to improve the quality of life, especially of elderly people.

Several phenomena take place during ageing, among them a low-level systemic inflammation (Ale and Binetti, 2021). Also the composition of microbiota changes with age, resulting in a gradual process that is influenced by several factors, like gender, location, diet, lifestyle, physical activity, immune system functionality, and the use of medication (O'Toole and Jeffery, 2015). The gut microbiota of elderly is affected by a shift toward a reduced bacterial diversity, characterized by a decline in beneficial microorganisms and an increase of facultative anaerobic bacteria (Salazar et al., 2017) (Slide 3). Considering that the inflammatory status of elderly population can be modulated by the gut microbiota and eating behaviours, the consumption of probiotic functional foods represents an interesting strategy (Guigoz et al., 2008; O'Toole and

Claesson, 2010). Probiotics may exert several effects on gut microbiota, manipulating and restoring its balance in richness and diversity. A “healthy” gut microbiota can impart specific functions in host: nutrients and drug metabolism, maintenance of gut mucosal barrier, immunomodulation, protection against pathogens, and production of metabolites (Figure 7; Slide 5).

Especially in elderly, the concept of healthy gut microbiota must be considered. According to this, the first habit to adopt is to choose a varied and well-balanced diet. Foods should be carefully selected paying attention to their quality. In addition, the introduction of probiotics and prebiotics can be of great help.

Probiotics can be consumed as capsule, supplement, or probiotic functional foods (chocolate bars, fruit juice, yogurt, ricotta cheese). The last option can be easily introduced in the daily diet, and it is generally well accepted by the consumers. Among the beneficial effects of probiotics, there are a good intestinal transit, a reduced sense of swelling, and an improved immune system (Slide 9).

Prebiotics, substances naturally present in foods, represent a real nourishment for gut microbiota. An example of prebiotic foods is those containing high amount of fibres, such as fruits and vegetables. These foods are also rich in antioxidants, that counteract the excess of free radicals, contributing to diseases prevention (Slide 6).

Figure 7. Example of slide from "Probiotics and Prebiotics".

9.3.1.6 Educational activity survey

When the nutritional education program was carried out, two different questionnaires were prepared and administered to the elderly subjects (Annex 6) and to the caregivers (Annex 7) of the boarding homes "A. Chierichetti" in Gagliole (MC) and “ASP Lazzarelli” in San Severino Marche (MC). The questionnaires were developed to have feedback about the educational activity proposed, in terms of understandability, learning and efficacy of this new approach. Each questionnaire was composed by 12 or 13 questions about the activity performed. That one addressed to the elderly subjects was more specific about the impact and effectiveness of the communication method used, while the questionnaire for the caregivers was more focused on the importance of personalized nutrition and supplementation in elderly subject for what concern their experience.

9.4 RESULTS

9.4.1 Nutritional education activities: video-pills

9.4.1.1 Elderly subject participants

This pilot project involved 16 elderly volunteers living in the two selected boarding homes. Females were 11, while males were five. Their mean age was 82.7 years old, and this aspect could represent a limit for this kind of learning activity. Overall, the videos and the practical activities proposed were really appreciated.

9.4.1.2 Elderly subjects survey

The questionnaire addressed to the elderly volunteers was organized into 13 questions, 7 of them were referred to the videos proposed, investigating the impact of this new strategy on subjects. The results are presented in Figure 8. A consensus among the participants was observed, since 62% of people recognized this approach as valid and useful. This was also confirmed by the interest of 69% of people to explore other topics using the same method, and 94% of them was available for further training activities. In general, the video-pills strategy was well accepted and easy to follow by 94%. Moreover, it stimulated a positive exchange of opinions, suggesting sharing these videos with relatives.

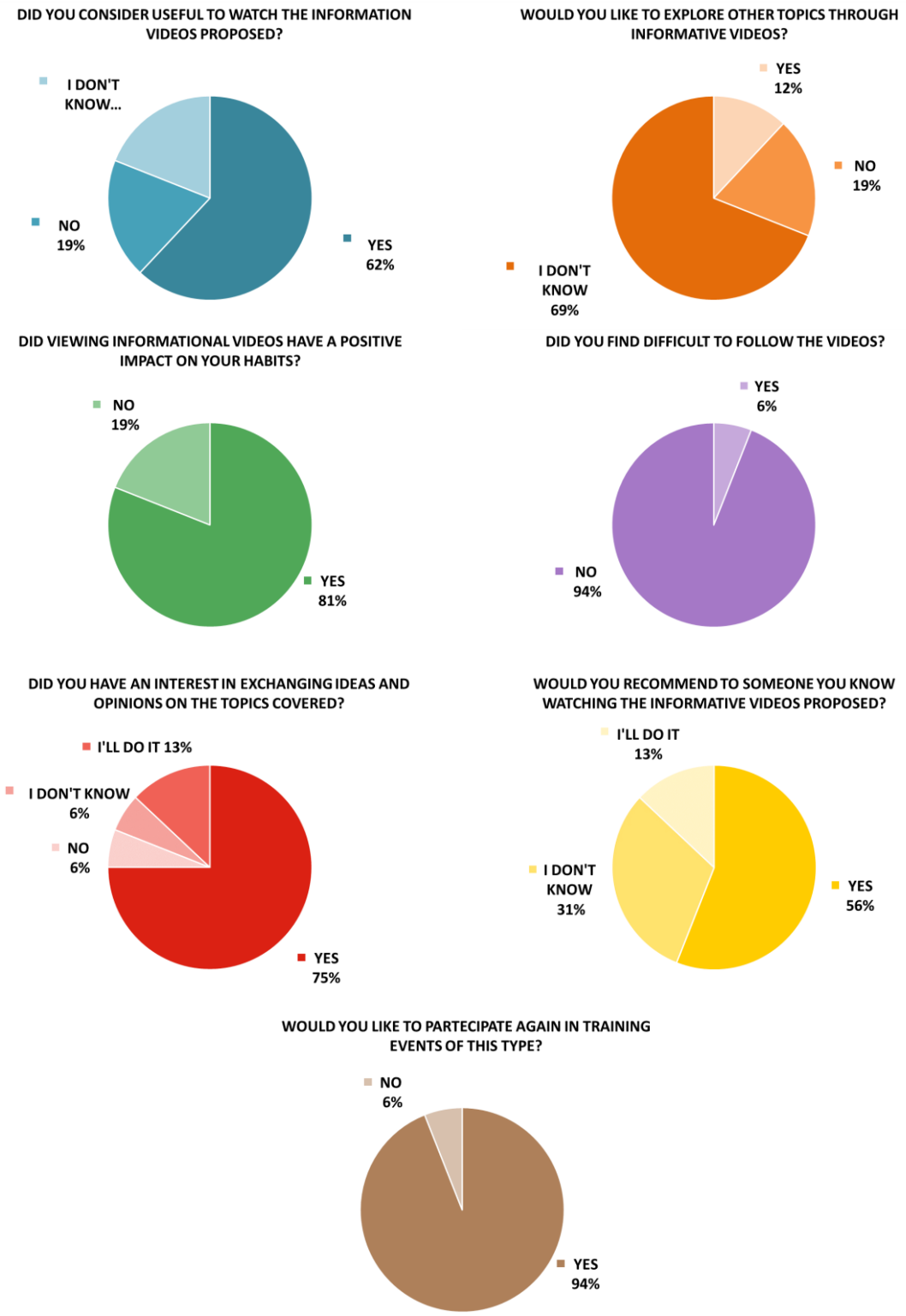

Figure 8. The impact of the teaching method on the elderly subjects.

Other five questions were focused on the effectiveness of the method, investigating the concepts that people learnt and remembered. Promising feedbacks were collected (Figure 9).

The 88% of subjects recognized the importance of a balanced and complete diet; the 44% understood the right amount of water to drink per day and the best strategies to adopt to remain hydrated; the 69% recognized the importance to vary the diet, choosing all the colours of fruits and vegetables. The level of agreement for the consumption of three complete meals per day is 69%, but only the 13% already had this daily habit. Since the 12% did not agree with this point, highlighting the need to improve this issue.

Surprisingly, the 75% of elderly subjects understood the beneficial effects of probiotics and prebiotics, while the 25% still had doubts.

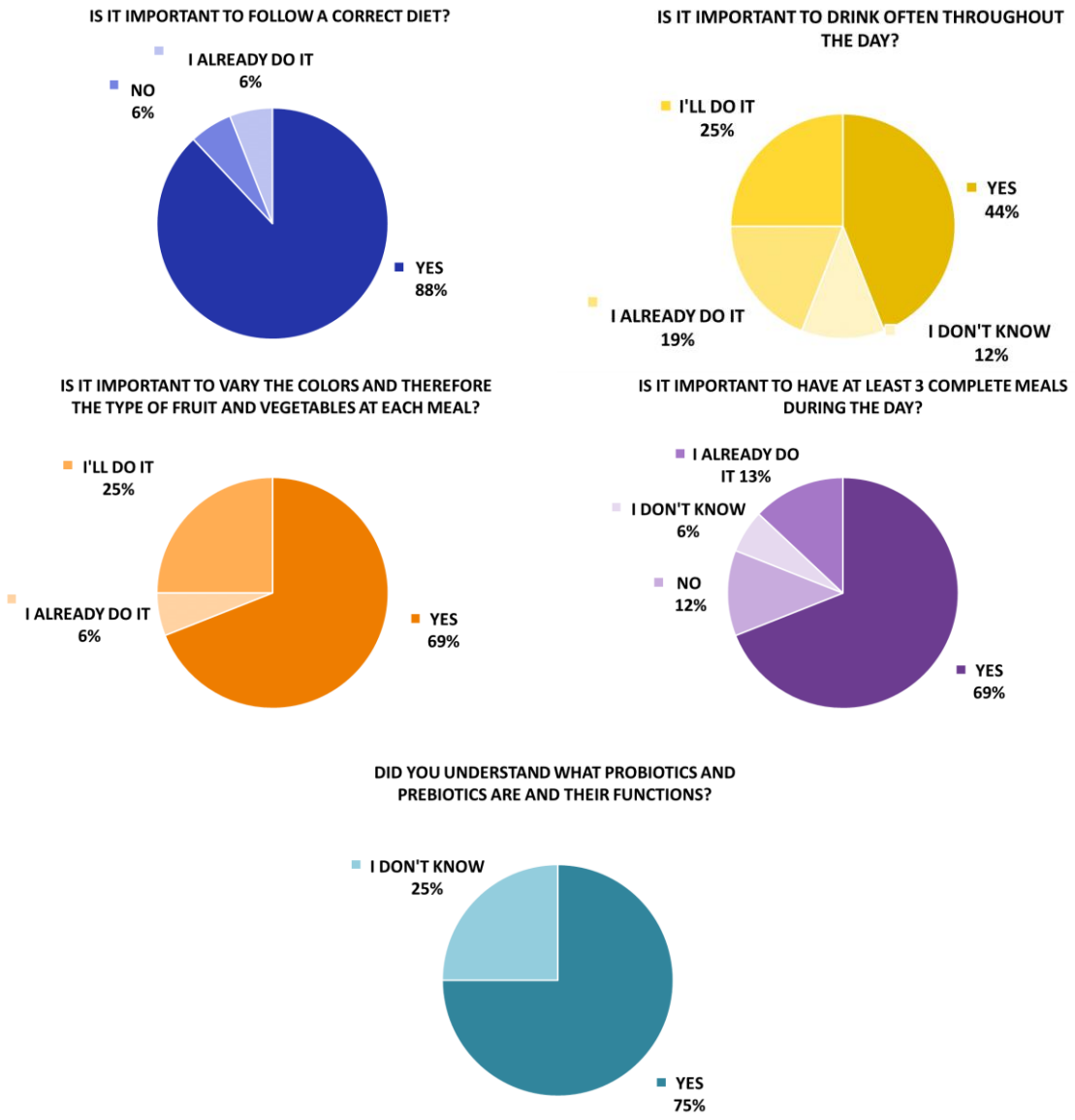

Figure 9. The efficacy of the method as learning tool.

The last question was about the PROBIOSENIOR project (Figure 10). The elderly subjects enrolled weren't aware about the type of supplementation they received. The feedback obtained investigating the beneficial effects from probiotic functional foods on the general well-being was 50%/50%, confirming the absence of negative effects on the subjects.

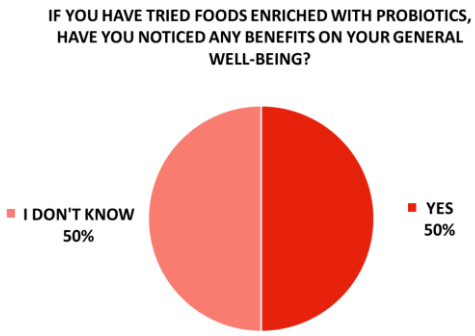

Figure 10. PROBIOSENIOR project impact on elderly.

9.4.1.3 Caregivers survey

The caregivers were also involved in this nutritional educational activity. An heterogenous staff of 17 females and two males, with a mean age of 42 years old, was recruited to view the videos and reply to the questionnaire.

The latter was organized in 12 questions. Six of them were referred to their personal and professional experience, other four investigated the impact of PROBIOSENIOR project, and the last two were focused on the effectiveness of the video-pills as teaching/learning method. The collected results are presented in Figures 11 - 13.

The answers revealed that all caregivers recognized the importance of nutrition in the daily treatment of patients. From a practical point of view, the 68% of them noticed differences adopting specific nutritional approach on patients with similar pathological status, while the 21% never thought about this aspect, and the 11% did not notice any difference. Concerning the nutritional status of the subjects hosted in the boarding homes, the 58% considered that there is mainly a normal nutritional status, while the 42% replied that malnutrition is prevalent. Similar conflicting answers were obtained about the specific diet administered to elderly subjects, basing on their pathological situation. The 74% affirmed that the diet is personalized, while the 26% did not confirm. The different answers collected were probably due to the different boarding homes where the caregivers worked.

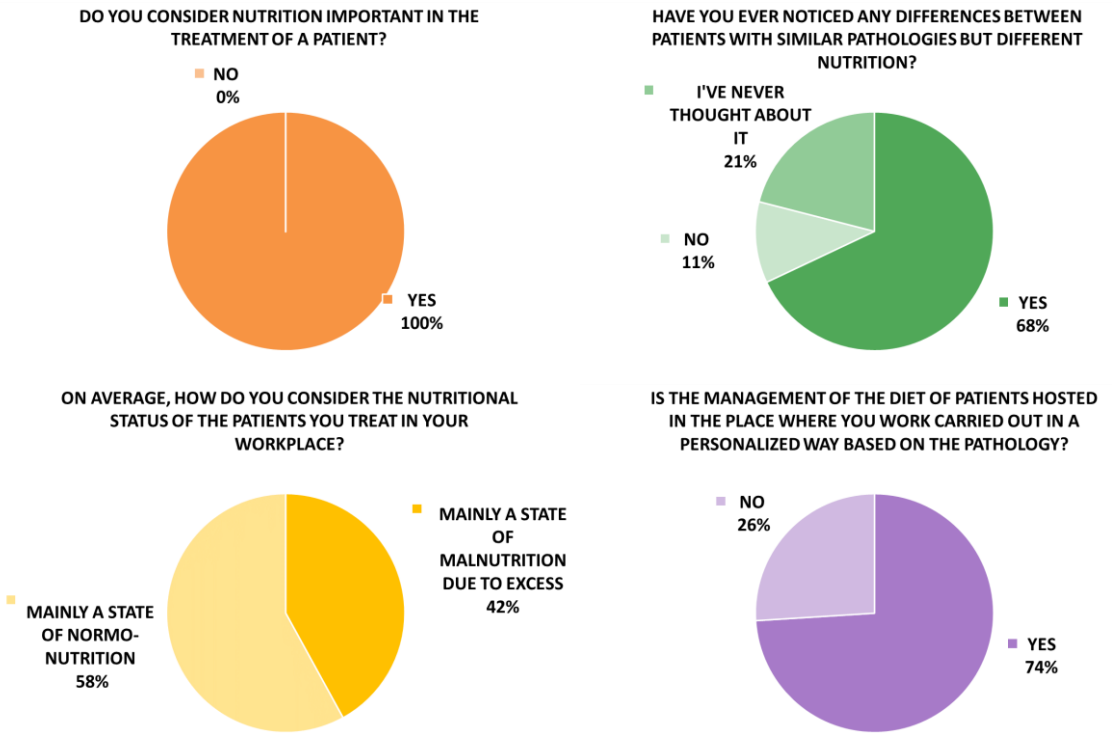

Figure 11. Personal and professional experience of caregivers.

In general, PROBIOSENIOR project had a positive impact on the caregivers population, as shown in Figure 12. Most of them (42%) already had experience with probiotics and prebiotics, while the 58% was not aware about their functions. Moreover, the 47% observed an improvement in elderly subject well-being after the probiotic supplementation, but the 53% did not notice.

The supplementation was administered in a satisfying and sustainable way for 79% of caregivers, while it can be improved for the 21% of them. Confirming this positive feedback, the 79% wanted to recommend these probiotic functional foods supplementation to third parts.

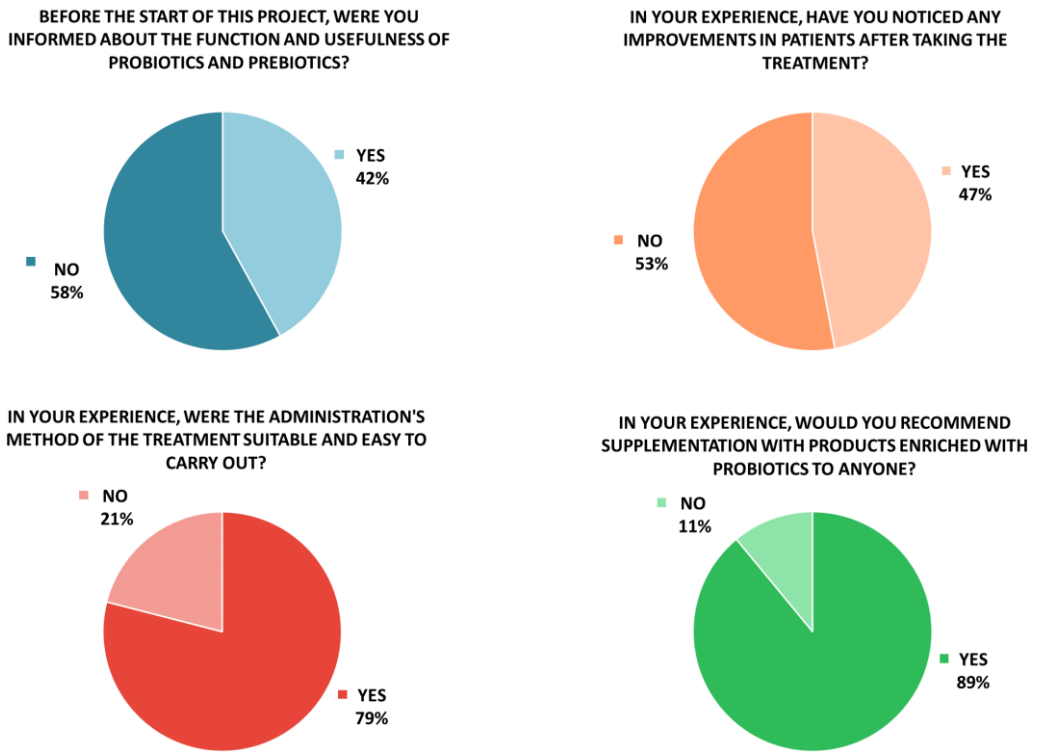

Figure 12. PROBIOSENIOR project impact on caregivers.

Figure 13 shows the impact of the video-pills method as innovative learning tool. The 74% of the caregiver staff believed that the videos are an efficient strategy and a good alternative to a live learning experience. They also affirmed that this kind of activity may help to increase the awareness of listeners. On the contrary, the 26% of caregivers did not believe in the effectiveness of the communication's method used for this audience.

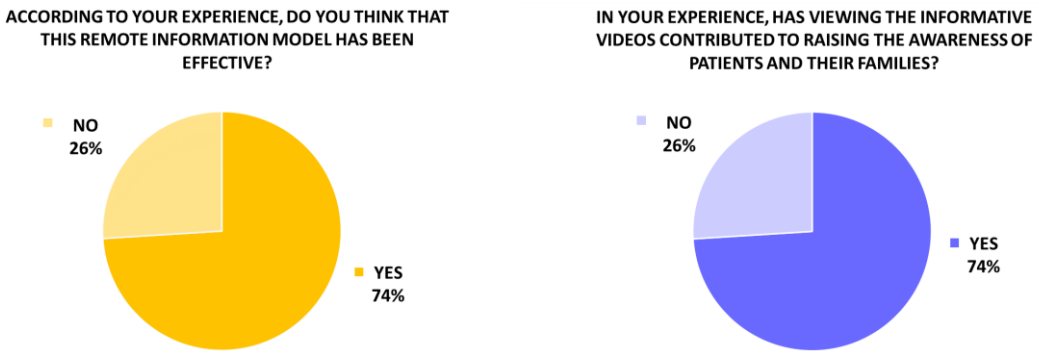

Figure 13. The efficacy of the used method as learning tool.

9.5 DISCUSSION AND CONCLUSIONS

Healthy diets have been demonstrated to play a key role in the health condition, influencing physical activity, physical status and mental well-being. All these aspects affect the quality of life of elderly people (Rusu et al., 2020). Moreover, the difficulties that older adults have in managing nutrition and food safety by themselves contribute to the increased incidence of malnutrition and a series of related conditions (Choi et al., 2016).

According to the evidence and the purpose of PROBIOSENIOR Project, a collateral activity was developed to increase the awareness on health and balanced diet, promoting healthy ageing.

The proposed nutritional educational intervention aimed to increase knowledge, confidence, and motivation to make changes in elderly people. This pilot project involved only two boarding homes, to assess its feasibility and effectiveness. The program was carried out using videos displayed in remote telematic mode, due to Covid-19 health emergency and relative restrictions. It was a double challenge considering the high average age of people involved.

Firstly, this innovative communication method was evaluated as a valid alternative to the live teaching approach. The 94% of the elderly expressed the desire to participate again to similar activities, supporting the effectiveness of this method even on older people.

The success of this activity was also confirmed by most seniors who had a positive impact on the daily habits, adopting little suggested changes. In addition, positive feedback was expressed by caregivers (74%) who considered these videos as an effectiveness method to increase the awareness on daily nutritional habits. On the contrary, a percentage (26%) of care givers expressed concerns about using this method in elderly context.

The impact of PROBIOSENIOR Project on elderly was also evaluated. The 50% of the interviewed people recognized benefits after the supplementation, the remaining did not notice any changes. Similar results were obtained from caregivers' questionnaire; the 47% of them observed an improvement in elderly health status, while the 53% did not.

This cross-section evaluation presented some limitations, due to the individual

sensitivity and perception, the type of supplementation received (probiotic or placebo), and other personal conditions that increased the variability of the results.

This activity, proposed in parallel with PROBIOSENIOR project, certainly contributed to improve the knowledge on probiotics and their beneficial effects, in both elderly and caregivers. The key aspects identified by the participants were: high-quality education, plain and intelligible language, interactive format with practical activities, social experience, and small size groups. In conclusion, this pilot intervention could provide a basis for designing effective and measurable nutritional education programs for older adults.

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CHAPTER X

FINAL CONCLUSIONS

As in many Western countries, in Italy the elderly population has been increasing markedly. The proportion of elderly in Europe is expected to increase from 17% in 2010 to approximately 30% by the year 2060 (Eurostat, 2011). This demographic shift will most probably increase the number of people with age-related diseases and disabilities. It is crucial to find valid strategies that decrease morbidity by slowing down the ageing process and thereby increase the healthful ageing. Nutrition is absolutely one of the most effective strategies that can lead to healthy ageing, and it is a potential factor that can be changed by elderly people themselves.

The ageing process is associated with a chronic, low-grade inflammatory condition that is a well-established background in many age-related diseases (Franceschi and Campisi, 2014). Inflammageing in the elder population is considered a risk factor for the development of several pathologies and many recent studies investigate the use of various inflammatory biomarkers such as C-reactive protein (CRP), interleukin-6 (IL-6) and IL-1, and as predictors of physical and cognitive performance among elderly (Pepys and Hirschfield, 2003).

CRP plays important roles in inflammatory and disease processes and host responses to infection, and the normal levels increase with ageing. Its elevated levels in serum have been identified to accompany increased vulnerability for disease and mortality in older patients, such as an increased risk of sarcopenia, cardiovascular pathologies, disability and cognitive decline in individuals over 65 years old (Velissaris et al., 2017).

Furthermore, older adults have a high prevalence of comorbid disease and concomitant exposure to different medications, including antibiotics. The inflammageing, through changes in lifestyle and age-related modifications in intestinal physiology, such as impaired dentition and salivary function, decreased motility with constipation, diverticular disease and dietary changes, profoundly affects also the homeostasis of the gut microbiota (GM) (Zapata and Quagliarello, 2015).

The intestinal microbiota, in eubiosis condition, plays a key role in host`s wellbeing, providing nutritional, metabolic and immunological benefits such as the synthesis of folate and vitamin B12, two critical vitamins in the elderly. The composition and abundance of individual GM changes until adulthood, but a major shift in its composition, called dysbiosis, can trigger harmful local and systemic inflammation, and also pathological conditions (Ragonnaud and Biragyn, 2021). The change of some key GM members, as *Clostridium coccoides* and *Eubacterium rectale* groups and *Clostridium* cluster IV, is associated with the depletion in butyrate and in an overall reduction of short chain fatty acids (SCFAs) in the gastrointestinal (GI) tract.

SCFAs, mostly acetate, butyrate and propionate, are microbiota metabolites representing an important energy source for the host, but of note they can modulate the immune functions and concur to maintain a functional GI epithelial barrier (Mäkivuokko et al., 2010). Consequently, the reduction of them can involve nutritional and immunological effects, contributing to the health impairment of older people; moreover, the increase in pro-inflammatory Enterobacteriaceae and the concomitant decrease in anti-inflammatory GM components result in the establishment of a pro-inflammatory microbial community, which can support the inflammageing process (Biagi et al., 2012; Rampelli et al., 2013).

In this scenario, the microbiome manipulation of elderly adults could be a parallel, innovative strategy to influence the development of ageing-associated comorbidities (Zapata and Quagliarello, 2015).

The aim of our double-blind, placebo-controlled study was to investigate the effects of a probiotics-based diet on the low-grade inflammation in clinically healthy older people. Moreover, PROBIOSENIOR project wants to develop a platform that enables the use of new functional foods and nutraceuticals containing probiotics, made with innovative technologies.

Different probiotic products are already in the market, but none is specific for elderly and above all, they do not have an innovative distribution, as proposed by this project. Further innovation factor is the experimental research phase to realize functional foods enriched with appropriate amount of high quality, patented probiotic bacterial strains to guarantee maximum functionality.

The main objectives of this study were to assess the effects of probiotic supplemented diet on plasma concentration of high-sensitivity C-reactive protein (hsCRP), GUT microbiota modulation, and consequently SCFAs production. Furthermore, the effects of probiotics supplementation on 11 biogenic amines were assessed and the gene expression pattern of TNF-α and IGF-1 was also investigated, followed by the quantitative determination of the circulating level of IGF-1 in the elderly subjects.

MAIN FINDINGS

Some interesting findings have been demonstrated.

1. As supported by literature, a significant proportion of the elderly people under study have had elevated HsCRP concentrations (Imhof et al., 2003). HsCRP is recognized as marker of low-grade inflammation, but also of cardiovascular risk, myocardial infarction and stroke (Pearson et al., 2003). In our study, the difference occurred from the T1 and T0 was significantly higher after probiotic intervention than the placebo (P<0.05). In addition, a further subgroup analysis, the 6 months' probiotic supplementation showed a significant effect (P<0.05) on the protein level respect to the shorter one (less than six months). This finding suggests that prolonged intervention is required to counter a chronic situation as in inflammageing process elderly associated. The older subjects might need longer treatment to achieve a gut microbiota stabilizing effect.

2. Another parameter strongly related to inflammageing is GUT microbiota. The gut bacterial composition of the elderly is characterised by a decrease in *Bacteroides* spp*.,* *Bifidobacterium* spp. and *Lactobacilli* spp. (Odamaki et al., 2016), but in our study, the populations of *Lactobacillus* spp. significantly increased after probiotic treatment. This positive result is probably due to the specific supplementation used. In addition, *Bifidobacterium* spp. increased in probiotic group after supplementation, though the improvement is not statistically significant. Finally, the prevalence of *Clostridium difficile*, a major intestinal pathogen, dramatically increases in the elderly, probably due to the antibiotic usage. We recorded the reduction of cell count of *Clostridium coccoides-Eubacterium rectale* group after both supplementations, with no significant differences for the treatment duration; but for *Staphylococcus* spp., a significant reduction (respect to baseline data) was demonstrated in the probiotic group.

No statistically significant difference in the α- and β-diversity in the samples of our study during the treatment was observed but this was not surprising. Intestinal microbiota tends to be quite stable during time in healthy subjects and it seems to have a documented resilience to perturbations. Moreover, the high inter-individual variations in the gut microbiota may have masked changes due to probiotic intake. Nevertheless, in the present study, a specific effect of the probiotic treatment was observed probably related to the long-term probiotic diet.

Results obtained with 16S NGS analysis revealed that the probiotic administration significantly affected some phyla, families and genera abundance. In detail, we observed a significant increase of Bifidobacteriaceae family and *Bifidobacterium* genus, that is inversely correlated with inflammageing and consequent ageing-associated morbidity and mortality (Mueller et al., 2006). We also found a significant increase in *Akkermansia* spp., a mucin-degrading microbe that provides energy to other beneficial microbes, and it is considered a biomarker for healthy ageing (Biagi et al., 2016).

3. Ageing is strongly associated with a progressively and significant reduction of SCFAs in the GI tract. In our study, considering just the supplementation type, no significant changes occurred in probiotic group with respect to placebo, after the supplementation. Nevertheless, comparing the level of SCFAs, in the same group at different time points (T0 vs T1), a significant increase was observed in the probiotic group only. Alterations in the abundance of the gut bacteria mediated by probiotic treatments might change metabolic ability as well.

4. Biogenic amines (BAs) are molecules that may be useful to evaluate health status of people, especially in old age, since they are involved in essential physiological functions and are used as markers of important diseases.

In our study, the effects of probiotics supplementation on 11 BAs were assessed. A high inter and intra-variability was observed at T0 and T1. The levels of some polyamines positively changed after probiotic supplementation respect to placebo; other BAs remained stable, and others were not detected because out of ranges of detection and quantification. Overall, the effect of probiotic supplementation was demonstrated as a trend influencing some BAs more than others. Further investigations are necessary to better understand the mechanisms behind the BAs modulation and its potential role in healthy ageing.

In elderly, inflammageing is associated also with a dysregulation of the immune system, leading to an increase in the concentration of pro-inflammatory cytokines, including tumour necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 (IL-1) as markers of inflammageing and age-related diseases and disability. Moreover, a marker of longevity seems to be Insulin-like growth factor 1 (IGF-1). In our study, we focused on the gene expression pattern of TNF-α and IGF-1. Firstly, we recorded a significant increase in circulating levels of IGF-1 in probiotic group, respect to placebo-supplemented one. As second findings, TNF-α levels and its gene expression resulted partially influenced by the dietary intervention. Overall, the data analysed indicate that the probiotic supplementation used can modulate the GH/IGF-axis throughout GUT microbiota modulation, with positive effects on the pathogenesis of sarcopenia and other age-related morbidities.

In addition to hsCRP levels, also general haematological parameters were measured to monitor health status of elderly throughout the study. The probiotic administration did not contribute to significant changes in blood parameters, but a promising improving in lipid profile was observed.

5. Among all available multidimensional tools, we used questionnaires as instruments to investigate the elderly population, living in boarding homes and in private houses. We evaluated all its aspects, including the lifestyle and eating habits, the nutritional status, the intestinal regularity, the physical and psychological health, the pathological anamnesis and pharmacological therapy. Our results revealed noteworthy positive improving in nutritional status and psychological well-being, after probiotic supplementation. On the other hand, lifestyle and eating habits, and the pathological conditions did not show relevant changes after both supplementations. In conclusion, our results suggest the apparent benefits of probiotic supplementation on psychological well-being and nutritional status, improving the quality of life in elderly.

The prevention program was a further effective instrument applied in this study. Considering the importance of the diet and the difficulties to manage the nutrition according to the individual energy requirements and physio-pathological status, we developed nutrition educational activities to improve healthful ageing, preventing malnutrition and relative morbidities. The program involved the elderly community of two boarding homes, and the caregivers. Firstly, the survey revealed that most people

recognized a positive impact on their daily nutritional habits. Moreover, this communication method resulted effective for elderly. Overall, this intervention certainly improved the knowledge and awareness on nutrition and probiotics, in both elderly and caregivers, suggesting the importance to develop specific preventing programs.

CONCLUSIONS

Although the two bacterial strains making up the blend were isolated from the intestine of elderly subjects, it is hard to define a typical gut microbiota composition of elderly (Silvi et al., 2003; An et al., 2018). The high variability of elderly condition and all factors influencing it may represent a limit in several studies.

Despite all, PROBIOSENIOR demonstrated to be an ideal support for healthy ageing and may have a significant impact on the social-health system. The 40% of the general population in Western countries is affected by functional disorders such as dyspepsia and irritable bowel syndrome (IBS), which includes constipation, bloating, impaired digestion, digestive pain and intermittent diarrhoea. Combining the knowledge about the influence that diet has on ageing and its potential role to prevent age-related diseases, the way will open for making the microbiota the target of intervention to improve the wellbeing of the elderly.

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List of publications

1. **Salvesi C.**, Silvi S., Fiorini D., Scortichini S., Sagratini G., Palermo F., De Leone R., Egidi N., Fatone L., Cifani C., Amedei A., Scocchera F., Morici M., Gatto B., Mannucci F., Valeriani V., Malavasi M., Servili S., Casula A., Cresci A., Corradetti I., Carpi F., Picciolini M., Coman M.M., Verdenelli M.C. (2022). Impact of a probiotic diet on wellbeing of healthy senior: THE PROBIOSENIOR PROJECT. *Journal of Applied Microbiology*. 00:1–13. https://doi.org/10.1111/jam.15747.

2. Coman M.M., Miorelli L., Micioni Di Bonaventura M.V., Cifani C., **Salvesi C.**, Amedei A., Silvi S., Verdenelli M.C. (2022). Effects of probiotic *Lactiplantibacillus plantarum* IMC 510 supplementation on metabolic factors in otherwise healthy overweight and obese individuals. *Journal of Applied Microbiology*. 133:1956–1968. https://doi.org/10.1111/jam.15703.

3. **Salvesi C.**, Silvi S., Fiorini D., Scortichini S., Sagratini G., Palermo F., De Leone R., Egidi N., Fatone L., Cifani C., Amedei A., Scocchera F., Morici M., Gatto B., Mannucci F., Valeriani V., Malavasi M., Servili S., Casula A., Cresci A., Corradetti I., Carpi F., Picciolini M., Coman M.M., Verdenelli M.C. (2022). Nutritional assessment and monitoring of biogenic amines and circulating factors in elderly subjects on probiotic supplementation (in preparation).

4. **Salvesi C.**, Coman M.M., Fiorini D., Silvi S. (2022). *In vitro* study of potential prebiotic properties of monovarietal extra virgin olive oils. Food Res. (submitted).

Contribution to conferences and workshops

1. **Salvesi C.**, Chen H., Mozzicafreddo M., Fiorini D., Attili A.R., Miceli C., Silvi S. (2021). Potential probiotic characterization of strains isolated from gut microbiota of Chinese young adults. **Poster** at virtual congress 12th International Symposium on Gut Microbiology, INRAE and Rowett Institute Organizing Committee, 13-15 October 2021.

2. **Salvesi C.**, Scortichini S., Caprioli G., Fiorini D., Silvi S. (2021). *In vitro* assessment of prebiotic effect of coffee and its by-products on human intestinal microbiota. **Oral presentation** at virtual congress Alimenti e Nutraceutici: salute e prevenzione attraverso il cibo. 5° Convegno Piattaforme Tematiche di Ateneo su “Alimenti e Nutrizione” e “Salute Umana e Animale”, 13 July 2021, Camerino, Italy.

3. Oral comunication “Cibo in fermento” at the online seminar “Conversazioni in fermento” organized by Slow Food Ancona and Conero Association, 29 March 2021.

4. **Salvesi C.**, Huang X., Fanizzi M., Scipioni T., Silvi S. (2020). Potential Effects of Commercial Kefir Milk Consumption on Gut Microbiota in Cancer Patients. **Poster** at

14th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health – Virtual International Probiotic Conference (IPC2020) 11 November 2020, Prague, Czech Republic.

5. Lenti L., Scortichini S., **Salvesi C.**, Chen H., Miceli C., Silvi S., Fiorini D. (2020). Short-chain fatty acids as biomarkers monitoring diet and environment effect on gut microbiota. 6th International Conference on FoodOmics, 14-16 October 2020, Cesena, Italy.

6. "Discovering Probiotics' World" at the online seminar organized by School of Advanced Studies (University of Camerino, Camerino, Italy) 08 May 2020.

7. **Salvesi C.** (2020). Probiotic functional foods-based diet for healthy ageing. **Oral presentation** at the NUTRITION WINTER SCHOOL 2020, Diet & Microbes: Gut health for the brain and body, 27-31 January 2020, University of Helsinki, Levi, Finland.

8. **Salvesi C.**, Verdenelli M.C., Fiorini D., Silvi S. (2019). Elderly people intestinal microbiota as target for probiotic functional food intervention. **Poster** at 10th Probiotics, prebiotics & new foods, nutraceuticals and botanicals for nutrition & human and microbiota health Congress, 08-10 September 2019, Rome, Italy.

9. **Salvesi C**., Miceli C., Silvi S. (2019). Characterization of novel probiotics from gut microbiota of chinese young adults. **Poster** at Cibo e nutraceutici: parola chiave Caratterizzazione. 4° Convegno Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale", 09 July 2019, Camerino, Italy.

10. Scortichini S., **Salvesi C.**, Silvi S., Fiorini D. (2019). Sviluppo e validazione di un metodo rapido e semplice per la quantificazione di acidi grassi a corta catena in campioni fecali e di fluidi di fermentazione. **Poster** at TUMA, 2019, Ancona, Italy.

11. **Salvesi C.** and Silvi S. (2019). Novel probiotics and postbiotics from gut microbiota of Chinese young adults. **Poster** at MICROBIOLOGY 2019 - Congress of SIMGBM, 19-22 June 2019, Florence, Italy.

12. Serri E., **Salvesi C.**, Pierantoni P., Gardi T., Cerquetella M., Galosi L., Silvi S., Rossi G. (2019). Preliminary evidence of gut microbiota composition of a particular population of honey bee from Marche region. **Poster** at Honeybee Health Symposium – New approaches to honey bee health, 13-15 February 2019, Rome, Italy.

13. **Salvesi C.**, Coman M.M., Fiorini D., Silvi S. (2018). *In vitro* study of potential prebiotic properties of extra virgin olive oils in cultivars from the Marche region. **Poster** at Cibo e nutraceutici: direzione salute. 3° Convegno a cura delle Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale", 10 July 2018, Camerino, Italy.

Courses, conferences and workshops attendances

13/10/2021-15/10/2021
12th International Symposium on Gut Microbiology, INRAE and Rowett Institute Organizing Committee (online).

12/09/2021-14/09/2021
11th Probiotics, prebiotics & new foods, nutraceuticals and botanicals for nutrition & human and microbiota health Congress, Rome, Italy.

13/07/2021
Alimenti e Nutraceutici: salute e prevenzione attraverso il cibo. 5° Convegno Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale", Camerino, Italy.

29/03/2021
Seminar "Conversazioni in fermento", Slow Food Ancona and Conero Association (online).

11/11/2020
14th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health – Virtual International Probiotic Conference (IPC2020).

08/05/2020
Online seminar organized by School of Advanced Studies (University of Camerino, Camerino, Italy).

27/01/2020-31/01/2020
NUTRITION WINTER SCHOOL 2020, Diet & Microbes: Gut health for the brain and body, University of Helsinki, Levi, Finland.

08/09/2019-10/09/2019
10th Probiotics, prebiotics & new foods, nutraceuticals and botanicals for nutrition & human and microbiota health Congress, Rome, Italy.

09/07/2019
Cibo e nutraceutici: parola chiave Caratterizzazione. 4° Convegno Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale", Camerino, Italy.

19/06/2019-22/06/2019
MICROBIOLOGY 2019 - Congress of SIMGBM, Florence, Italy.

27/03/2019
Training course "Trucchi e segreti della GC Capillare", Phenomenex, Milano, Italy.

10/07/2018
Cibo e nutraceutici: direzione salute. 3° Convegno a cura delle Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale", Camerino, Italy.

12/09/2021-14/09/2021
11th Probiotics, prebiotics & new foods, nutraceuticals and botanicals for nutrition & human and microbiota health Congress, Rome, Italy.

International Research Experience

From January to February 2022 – Visiting research secondment at Microbiology Laboratory, University College Cork (Cork, Ireland). *Reference: Dr. Cormac Gahan.*

Scholarship and competitions

Winner of scholarship for NUTRITION WINTER SCHOOL 2020, Diet & Microbes: Gut health for the brain and body, University of Helsinki, Levi, Finland (January 2020).

Academic Activities

Placement Tutor of Final Reports of Bachelor's Degree students

1. Enumeration of bacterial groups from elderly faecal samples. Stage of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY. Candidate: Gideon Baah Boahen. University tutor: Prof. S. Silvi; Placement tutor: Dr. C. Salvesi (waiting for graduation).

2. Evaluation of probiotic properties of *Weissella paramesenteroides* isolated from fermented food. Final Report of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY, Curriculum: Biotechnology. Candidate: Vita Zaccaria. University tutor: Prof. S. Silvi; Placement tutor: Dr. C. Salvesi. Academic Year: 2020-2021.

3. Isolation and characterization of probiotic properties of bacterial strains from honeybee gut. Final Report of Bachelor's degree in BIOSCIENCES and BIOTECHNOLOGY, Curriculum: Biology. Candidate: Martina Palmieri. University tutor: Prof. S. Silvi; Placement tutor: Dr. C. Salvesi. Academic Year: 2019-2020.

Placement Tutor of Final Reports of Master's Degree students

1. Isolation and characterization of new lactic acid bacteria strains from several biological matrices. Experimental thesis of Master's degree in BIOLOGICAL SCIENCES. Candidate: Keerthana Kaladharan. University tutor: Prof. S. Silvi; Placement tutor: Dr. C. Salvesi (waiting for graduation).

2. *In vitro* assessment of prebiotic effect of coffee bean and its by-products in human intestinal health. Experimental thesis of Master's degree in BIOLOGICAL SCIENCES, Curriculum: Molecular Diagnosis and Biotechnology. Candidate: Varsha Bhaskar. University tutor: Prof. S. Silvi; Placement tutor: Dr. C. Salvesi. Academic Year: 2019-2020.

Other activities

1. Member of exam Commission as Subject Expert on “General and Industrial Microbiology” (Bachelor Degree – Biosciences and Biotechnologies) and on “Functional Food" (Master Degree – Biological Sciences).

2. Teaching activities (within curriculum of Ph.D.) – International School of Advanced Studies (University of Camerino) at “Scienze gastronomiche” and “Laboratory II” (Microbiology module) within the Biosciences and Biotechnology Course.

3. Tutor activities for high school students in visit/stage in the Microbiology Laboratory.

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ANNEXES

Annex 1. Flyer of PROBIOSENIOR Project.

Annex 2. Lifestyle and eating habits questionnaire.

Personal data:

Age range:

- 65-70 years
- 70-75 years
- 75-80 years
- Over:
- Weight (kg):

Height (m):

Waist circumference (cm):

Lifestyle questionnaire

1) You live:

- alone
- with the spouse-cohabitant
- in family
- in a structure

2) Where is your house:

- urban centre
- town / village
- isolated

3) In case of need have you ever addressed to your neighbours?

- sometimes
- never
- Other:

4) Did you need home delivery?

- never
- to do the shopping / cooking
- for house cleaning
- for injections / dressings
- Other:

5) Have you ever had experience of delivery shopping / meals at home?

- Yes
- No
- If YES: have you been satisfied?
    - Yes

- No
- Other:

- If NO: would you like to try?
  - Yes
  - No
  - Other:

6) Would it be inconvenient or discomfort the weekly visit of a courier for the delivery of shopping / meals at home?

- Yes
- No

7) In which time slot would NOT want deliveries to occur? (because you are resting, eating, you usually come out ...)

- before 8:00
- from 8.00 to 12.30
- from 12:30 to 14:30
- from 2.30pm to 6.30pm
- after 6:30 pm

8) Are you doing any physical activity?

- Yes
- No

- If yes, what kind of activity (walking, cycling, dancing, etc.)? :
- How often?
  - everyday
  - from 1 to 3 times a week
  - rarely

9) Do you go out for lunch or dinner?

- Yes
- No

- How often?
  - 1 time a week
  - 1-2 times a month
  - rarely

10) Are you taking vitamin or mineral or dietary supplements?
(Indicate which vitamins or mineral salts or supplements you are taking):

11) Are you taking dietary supplements and food bars?

- Yes
- No, I stopped
- No

- If so: How do you evaluate the use of dietary supplements?:

- If you stopped: Why did you stop using dietary supplements?:

12) Are you taking drugs?

- Yes
- No
- If so:

  Do you take more than 3 kinds of drugs?
  - Yes
  - No

  Do you have difficulty remembering hiring schedules?
  - Yes
  - No

Eating habits

1) Do you suffer from any kind of intolerance and / or allergy?

- Yes
- No
- If so: to what?:

2) Are you following a particular diet?

- Yes
- No
- If so, which one:
  - vegetarian
  - vegan
  - gluten free
  - Other:

3) How many complete meals do you usually consume in a day?

- 2 meals
- 3 meals
- 4 meals
- Other:

4) How many times a week do you eat pasta or rice or pizza?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- Do you usually eat them more often:

  - at lunch
  - at dinner
  - for lunch or dinner
  - both for lunch and dinner

5) How many times a week do you eat bread / breadsticks / crackers?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - at lunch
  - at dinner
  - for lunch or dinner
  - both for lunch and dinner

6) How many times a week do you eat dairy products? (e.g. milk or cheese)

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - at lunch
  - at dinner
  - for lunch or dinner
  - both for lunch and dinner
- What are the dairy products you consume most often:
  - milk
  - yogurt
  - fresh cheeses
  - ripened cheese
  - Other:

7) How many times a week do you eat legumes or eggs?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - at lunch
  - at dinner

- for lunch or dinner
- both for lunch and dinner

8) How many times a week do you eat based-meat products?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - at lunch
  - at dinner
  - for lunch or dinner
  - both for lunch and dinner

9) How many times a week do you eat meat or fish?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - at lunch
  - at dinner
  - for lunch or dinner
  - both for lunch and dinner
- Between meat and fish you eat more frequently:
  - meat
  - fish
  - both of them

10) How many times a week do you eat sweets?

- everyday
- 4-6 days
- 2-3 days
- 0-1 days
- You usually eat them more often:
  - after the main meals
  - as a snack
- What are the desserts that you consume most often:
  - packaged biscuits / snacks
  - cakes / homemade pies or pies from the baker

- ice cream
- candies
- Other:

11) How many times a day do you eat fruit?

- never
- Other:
- You usually eat it more often:
  - after the main meals
  - as a snack
- What is the way to eat fruit that you prefer and / or consume more often?
  - fruit
  - cooked fruit
  - fruit pulp
  - centrifuged / smoothie
  - fruit juice
  - Other:

12) How many times a day do you consume vegetables?

- never
- Other:

13) How many glasses of water (alternatively Tea) do you drink per day?

- less than 3 glasses
- from 3 to 5 glasses
- more than 5 glasses

14) What other drinks do you have?

- fruit juices
- soda or sweetened drinks (coca cola, orange, lemon, cedar, etc)
- Other:

15) Do you have one or more snacks during the day?

- never / rarely
- in the morning
- in the afternoon
- both in the morning and in the afternoon
- What you usually consume during the snack:

16) Is the packaging of a product influencing the choice of a food or a brand, rather than another ?

- Yes
- No
- Indifferent

17) What kind of packaging do you prefer?

- single-dose
- family packs
- bulk food
- Other

Annex 3. Pathological anamnesis and Pharmacological therapy questionnaire

1) Alteration of lipid metabolism:

- High cholesterolemia or to take antidyslipidemic therapy
  - Yes
  - No
- High triglyceridemia or antidyslipidemic therapy
  - Yes
  - No

2) Alteration of glycaemic compensation

- High blood sugar (above 116 mg)
  - Yes
  - No
- Diabetes and/or to take oral therapy or insulin
  - Yes
  - No

3) Diseases of cardiovascular system

- Arterial hypertension or to take antihypertensive therapy
  - Yes
  - No
- Atherosclerotic disease (coronary heart disease, angina, heart attack, stroke, etc.)
  - Yes
  - No
- Cardiac insufficiency (breathlessness, sweating) during acts of daily life or at rest.
  - Yes
  - No

4) Respiratory app.

- Sleep apnoea (OSAS) or restrictive respiratory failure (COPD), documented with spirometry/cardiorespiratory monitoring/polysomnography
  - Yes
  - No
- Wheezing during moderate exertion (e.g. climbing a flight of stairs) or snoring
  - Yes
  - No

5) Skeletal app.

- Arthrosis (hip, knees, rachis) already documented with radiography or other investigation
  - Yes
  - No
- To use an aid.
  - stick
  - walker
  - pram
  - no

6) Genitourinary app.

- Polycystic ovary documented ultrasonically
  - Yes
  - No
- Stress incontinence (e.g. loss of urine during exertion or cough)
  - Yes
  - No

7) Gastroenteric App.

- Fatty liver, calculi of the cholecyst documented ultrasonically, gastritis, reflux
  - Yes
  - No

8) Neurological App.

- Parkinson
  - Yes
  - No
- Epilepsy
  - Yes
  - No

9) Surgery

- Have you undergone surgery? If yes, and in which year:
  - Other:
  - No

10) Family history

- Family history of premature cardiovascular disease (myocardial infarction, stroke, and/or sudden death before age 55 for the father or 65 for the mother)

- Yes
- No

11) Behaviour

- Major depressive disorder, bipolar disorder, schizophrenic disorder, borderline and antisocial personality disorders, panic and obsessive-compulsive disorders, certified by a physician, psychotherapist and/or psychiatrist
  - Yes
  - No
- Alterations of eating behaviour (stress eating)
  - Yes
  - No
- Eating Behaviour Disorders such as bulimia nervosa, nutritional feeding, nocturnal nutrition certified by a medical psychotherapist and/or psychiatrist
  - Yes
  - No

12) Others

- Have you 3 or more ambulatory weight loss programs failed?
  - Yes
  - No
- Have you already been admitted to the Nutritional Rehabilitation Unit in the past?
  - Yes
  - No
- Are you self-sufficient? if not specify what help is needed
  - Yes
  - Other:
- Need for oxygen therapy and / or respirator?
  - Yes
  - No
- Use of current or past substances. (If in the past indicate year)
  - No
  - Other:
- Abuse of wine or other alcoholic beverages (e.g. more than 3 glasses a day)?
  - Yes
  - No

Therapy in place

Indicate the drug name, daily dosage and motivation of the therapy in progress.

Annex 4. Mini Nutritional Assessment (MNA).

Mini Nutritional Assessment
MNA®

Nestlé Nutrition Institute

Last name: First name:

Sex: Age: Weight, kg: Height, cm: Date:

Complete the screen by filling in the boxes with the appropriate numbers.
Add the numbers for the screen. If score is 11 or less, continue with the assessment to gain a Malnutrition Indicator Score.

Screening

A Has food intake declined over the past 3 months due to loss of appetite, digestive problems, chewing or swallowing difficulties?
0 = severe decrease in food intake
1 = moderate decrease in food intake
2 = no decrease in food intake ☐

B Weight loss during the last 3 months
0 = weight loss greater than 3kg (6.6lbs)
1 = does not know
2 = weight loss between 1 and 3kg (2.2 and 6.6 lbs)
3 = no weight loss ☐

C Mobility
0 = bed or chair bound
1 = able to get out of bed / chair but does not go out
2 = goes out ☐

D Has suffered psychological stress or acute disease in the past 3 months?
0 = yes 2 = no ☐

E Neuropsychological problems
0 = severe dementia or depression
1 = mild dementia
2 = no psychological problems ☐

F Body Mass Index (BMI) (weight in kg) / (height in m^2)
0 = BMI less than 19
1 = BMI 19 to less than 21
2 = BMI 21 to less than 23
3 = BMI 23 or greater ☐

Screening score (subtotal max. 14 points) ☐☐
12-14 points: Normal nutritional status
8-11 points: At risk of malnutrition
0-7 points: Malnourished
For a more in-depth assessment, continue with questions G-R

Assessment

G Lives independently (not in nursing home or hospital)
1 = yes 0 = no ☐

H Takes more than 3 prescription drugs per day
0 = yes 1 = no ☐

I Pressure sores or skin ulcers
0 = yes 1 = no ☐

J How many full meals does the patient eat daily?
0 = 1 meal
1 = 2 meals
2 = 3 meals ☐

K Selected consumption markers for protein intake
- At least one serving of dairy products (milk, cheese, yoghurt) per day yes ☐ no ☐
- Two or more servings of legumes or eggs per week yes ☐ no ☐
- Meat, fish or poultry every day yes ☐ no ☐

0.0 = if 0 or 1 yes
0.5 = if 2 yes
1.0 = if 3 yes ☐,☐

L Consumes two or more servings of fruit or vegetables per day?
0 = no 1 = yes ☐

M How much fluid (water, juice, coffee, tea, milk...) is consumed per day?
0.0 = less than 3 cups
0.5 = 3 to 5 cups
1.0 = more than 5 cups ☐,☐

N Mode of feeding
0 = unable to eat without assistance
1 = self-fed with some difficulty
2 = self-fed without any problem ☐

O Self view of nutritional status
0 = views self as being malnourished
1 = is uncertain of nutritional state
2 = views self as having no nutritional problem ☐

P In comparison with other people of the same age, how does the patient consider his / her health status?
0.0 = not as good
0.5 = does not know
1.0 = as good
2.0 = better ☐,☐

Q Mid-arm circumference (MAC) in cm
0.0 = MAC less than 21
0.5 = MAC 21 to 22
1.0 = MAC 22 or greater ☐,☐

R Calf circumference (CC) in cm
0 = CC less than 31
1 = CC 31 or greater ☐

Assessment (max. 16 points) ☐☐,☐
Screening score ☐☐,☐
Total Assessment (max. 30 points) ☐☐,☐

Malnutrition Indicator Score

24 to 30 points	☐	Normal nutritional status
17 to 23.5 points	☐	At risk of malnutrition
Less than 17 points	☐	Malnourished

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1. Vellas B, Villars H, Abellan G, *et al*. Overview of the MNA® - Its History and Challenges. *J Nutr Health Aging*. 2006; **10**:456-465.
2. Rubenstein LZ, Harker JO, Salva A, Guigoz Y, Vellas B. Screening for Undernutrition in Geriatric Practice: Developing the Short-Form Mini Nutritional Assessment (MNA-SF). *J. Geront*. 2001; **56A**: M366-377
3. Guigoz Y. The Mini-Nutritional Assessment (MNA®) Review of the Literature - What does it tell us? *J Nutr Health Aging*. 2006; **10**:466-487.

For more information: www.mna-elderly.com

Annex 5. The Psychological General Well-Being Index (PGWBI).

1) In the last 4 weeks, how did you feel in general?

- In excellent spirits
- In very good spirits
- In good spirits mostly
- I have been up and down in spirits a lot
- In low spirits mostly
- In very low spirits

2) In the last 4 weeks, have you been bothered by diseases, physical disorders or pains?

- Every day
- Almost every day
- About half of the time
- Several times, but less than half the time
- Rarely
- Never of the time

3) In the last 4 weeks, have you felt depressed?

- Yes, to the point that I felt like taking my life
- Yes, to the point that I did not care about anything
- Yes, I felt very depressed almost every day
- Yes, I felt quite depressed several times
- Yes, I felt a little depressed sometimes
- No, I've never felt depressed

4) In the last 4 weeks, have you firm in control with your behaviour, thoughts, emotions and feelings?

- Yes, of course
- Yes, almost completely
- Yes, generally
- Not too much
- No, and I am somewhat disturbed
- No, and I am very disturbed

5) In the last 4 weeks, have you been bothered nervousness or your "nerves"?

- Extremely, so-to the point where I could not work or take care of things
- Very much so
- Quite a bit
- Some, enough to bother me

- A bit
- Not at all

6) In the last 4 weeks, how much energy or vitality did you have or feel?

- Very full of energy-very lively
- Quite full of energy most of the time
- I had significant ups and downs of vitality and energy
- My energy or vitality level was generally low
- My energy or vitality level has almost always been very low
- I felt powerless, emptied, devoid of energy or vitality

7) In the last 4 weeks, I felt discouraged and sad.

- None of the time
- A little of the time
- Some of the time
- A good bit of the time
- Most of the time
- All of the time

8) In the last 4 weeks, have you been generally tense or have you felt tension?

- Yes, I was extremely tense most or all the time
- Yes, I was very tense most of the time
- Generally not, but it happened to me several times to feel rather tense
- Sometimes I felt a little tense
- My level of tension was quite low
- I never had the feeling of being tense

9) In the last 4 weeks, how happy or satisfied or pleased have you been with your personal life?

- Really very happy-I could not have been more satisfied or pleased
- Very happy most of the time
- In general, very satisfied - pleased
- Sometimes fairly happy, sometimes fairly unhappy
- In general, dissatisfied or unhappy
- Almost always or always very dissatisfied or unhappy

10) In the last 4 weeks, have you felt so good to do what you wanted or had to do?

- Yes definitely
- Yes, to do almost everything I wanted or had to do
- My health problems have limited me to some important things

- Because of my health I am barely able to take care of myself
- I needed some help to take care of myself
- I needed help for everything or almost everything I had to do

11) Over the past 4 weeks, have you felt so sad, discouraged, hopeless or have had so many problems that you wondered if anything was worthwhile?

- Yes, enormously enough to the point that I have just about given up
- Very so much
- Quite a bit
- Yes, enough to bother me
- A little bit
- Not at all

12) In the last 4 weeks, I woke up feeling fresh and rested.

- Never
- Hardly ever
- A part of the time
- A lot of time
- Almost always
- Always

13) Have you felt apprehension, worry or fear for your health in the last 4 weeks?

- Extremely so
- Very mush so
- Quite a bit
- Some, but not a lot
- Practically ever
- Not at all

14) In the last 4 weeks, did you have any reason to wonder if you were losing your mind or if you were losing control of your memory, from the way you act, talk, think or feel?

- Not at all
- Only a little
- Some, but not enough to be concerned or worried about
- Some and I have been a little concerned
- Some and I am quite concerned
- Yes, very much so and I am very concerned

15) In the last 4 weeks, my daily life has been interesting for me.

- Never

- Hardly ever
- A part of the time
- A lot of time
- Almost always
- Always

16) In the last 4 weeks, have you felt active, strong or slow, lazy?

- Always very active and strong
- Almost always active and strong - never really slow and lazy
- Quite active and in force - rarely slow and sluggish
- Quite slow and lazy - rarely active and in strength
- Almost always slow and lazy - never really active and strong
- Always very slow and lazy

17) In the last 4 weeks, have you been anxious, worried or angry?

- Extremely, so much to feel bad or almost
- Very much so
- Quite a lot of
- Some, enough to bother me
- A little bit
- Not at all

18) In the last 4 weeks, I felt emotionally stable and sure of myself.

- Never
- A little of the time
- Some of the time
- A good bit of the time
- Most of the time
- Always

19) In the last 4 weeks, have you felt relaxed, ease or felt very tense, nervous or keyed up?

- Always relaxed and peaceful
- Almost always relaxed and peaceful
- Generally relaxed and calm, but sometimes quite tense
- Generally very tense, but sometimes quite relaxed
- Almost always very tense, nervous, or keyed-up
- Always very tense, nervous, or keyed-up

20) In the last 4 weeks, I felt happy and serene.

- Never
- Hardly ever

- A part of the time
- A lot of time
- Almost always
- Always

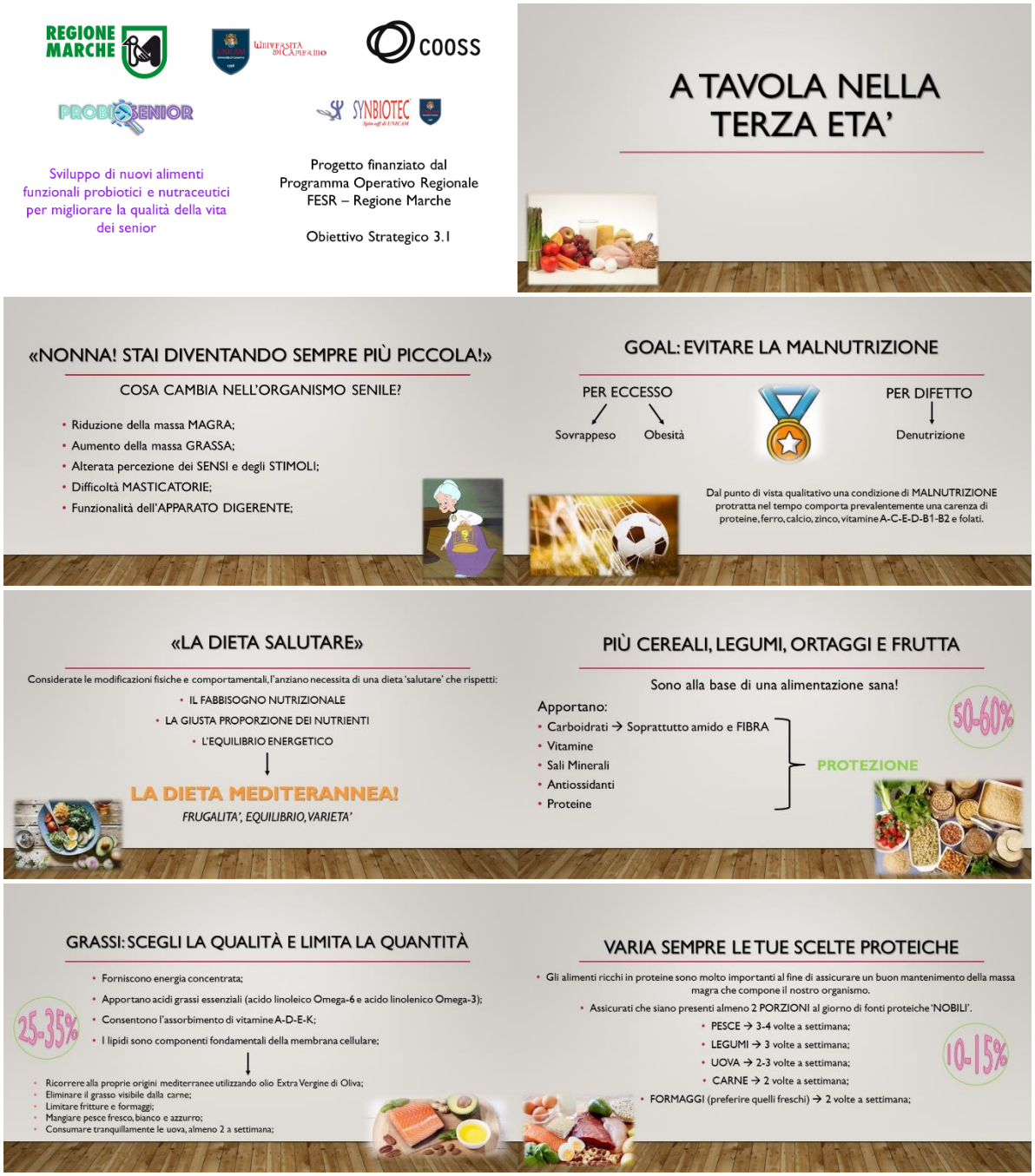
21) In the last 4 weeks, I felt tired, worn out, used up or exhausted.

- Never
- Hardly ever
- A part of the time
- A lot of time
- Almost always
- Always

22) In the last 4 weeks, have you been under or felt you have been under strain, stress or pressure?

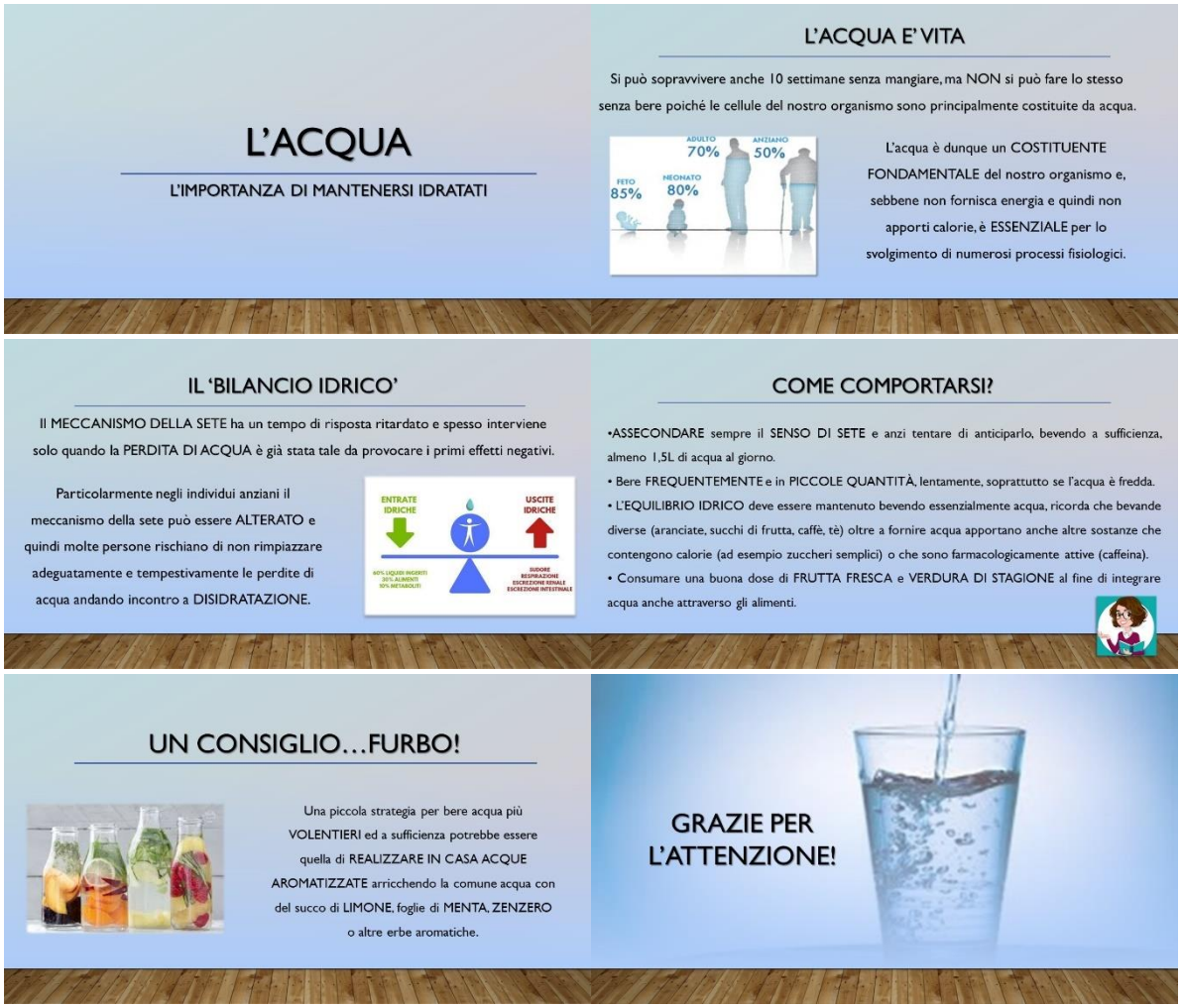
- Yes, almost more than I could bear or stand
- Yes a lot
- Yes, enough - more than usual
- Yes, enough - but almost usual
- Yes a bit
- Not at all

Annex 6. Presentation for the video "Nutrition for older adults".



(Deleuse M., 2020. Degree Thesis "Food habits improving strategies and monitoring some circulating factors in elderly subjects on probiotic supplementation")

Annex 7. Presentation for the video "The importance of water".



(Deleuse M., 2020. Degree Thesis "Food habits improving strategies and monitoring some circulating factors in elderly subjects on probiotic supplementation")

Annex 8. Presentation for the video "The healthy eating plate".

(Deleuse M., 2020. Degree Thesis "Food habits improving strategies and monitoring some circulating factors in elderly subjects on probiotic supplementation")

Annex 9. Presentation for the video "The colours of health".

(Deleuse M., 2020. Degree Thesis "Food habits improving strategies and monitoring some circulating factors in elderly subjects on probiotic supplementation")

Annex 10. Presentation for the video "Probiotics and prebiotics".

(Deleuse M., 2020. Degree Thesis "Food habits improving strategies and monitoring some circulating factors in elderly subjects on probiotic supplementation")

Annex 11. Questionnaire to the elderly subjects.

Sesso: …

Età: …

QUESTIONARIO SULLE ABITUDINI ALIMENTARI

(SCEGLIERE ANCHE PIU' DI UNA RISPOSTA PER OGNI DOMANDA LADDOVE OPPORTUNO)

Ha ritenuto di sua utilità la visione dei video informativi proposti?

Si

No

Non so

E' importante seguire una corretta alimentazione?

Si

No, non mi interessa

Controllavo già la mia alimentazione

Bere spesso durante la giornata, è importante?

Si

No

Lo facevo già

Lo farò

Non so

Variare i colori e quindi il tipo di frutta e verdura ad ogni pasto è importante?

Si

No

Lo facevo già

Lo farò

Non so

E' importante fare almeno 3 pasti completi durante la giornata?

Si

No

Lo facevo già

Lo farò

Non so

Ha compreso cosa sono i probiotici ed i prebiotici e le loro funzioni?

Si

No

Non so

Lo sapevo già

Qualora avesse provato degli alimenti arricchiti con probiotici ha notato dei benefici sul suo benessere generale?

Si

No

Non so

Le piacerebbe approfondire altri argomenti attraverso video informativi?

No

Non so

Si (specificare quali…)

Ha avuto difficoltà a seguire i video?

No

Si (specificare perché…)

La visione dei video informativi ha avuto un impatto positivo sulle sue abitudini?

No

Si

Ha avuto interesse a scambiare idee ed opinioni riguardo gli argomenti trattati?

No

Si

Non so

Lo farò

Consiglierebbe a qualcuno di sua conoscenza la visione dei video informativi proposti?

No

Si

Non so

Lo farò

Parteciperebbe nuovamente con piacere ad eventi formativi di questo tipo?

Si

No (spiegare perché…)

Annex 12. Questionnaire to the caregivers.

Sesso: …

Età: …

QUESTIONARIO

Esperienza lavorativa

Meno di 5 anni di servizio

Tra 5 e 10 anni di servizio

Tra 10 e 15 di servizio

Più di 15 anni di servizio

Stile di vita

Sedentario

Poco attivo

Attivo (pratico regolarmente attività fisica ≈ 3 ore a settimana)

Molto attivo (pratico attività fisica più di 3 ore a settimana)

Considera importante la nutrizione nel trattamento di un paziente?

Si

No

Non ci ho mai pensato

Ha mai notato delle differenze tra pazienti con patologie simili ma nutrizione diversa?

Si

No

Non ci ho mai pensato

Mediamente come considera lo stato nutrizionale della popolazione di pazienti che tratta nel suo luogo di lavoro?

Prevalentemente stato di malnutrizione per difetto

Prevalentemente stato di malnutrizione per eccesso

Prevalentemente stato di normo-nutrizione

La gestione della dieta dei pazienti ospitati nel luogo in cui lavora viene effettuata in maniera personalizzata in base alla patologia?

Si

No

Prima dell'inizio di questo progetto era informato riguardo la funzione e l'utilità di probiotici e prebiotici?

Si

No

Secondo la sua esperienza ha notato dei miglioramenti nei pazienti dopo l'assunzione del trattamento?

Si

No

Secondo la sua esperienza le modalità di fruizione del trattamento sono risultate idonee e di facile svolgimento?

Si

No (specificarne i motivi…)

Secondo la sua esperienza la visione dei video informativi ha contribuito alla sensibilizzazione dei pazienti e delle loro famiglie?

Si

No

Secondo la sua esperienza pensa che questo modello di informazione a distanza sia stato efficace?

Si

No

Secondo la sua esperienza consiglierebbe a qualcuno una integrazione con prodotti arricchiti con probiotici?

Si

No