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# In vitro and in vivo lipidomics as a tool for probiotics evaluation

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## Abstract

The probiotic bacteria are helpful for nutritional and therapeutic purposes, and they are commercially available in various forms, such as capsules or powders. Increasing pieces of evidence indicate that different growth conditions and variability in manufacturing processes can determine the properties of probiotic products. In recent years, the lipidomic approach has become a useful tool to evaluate the impact that probiotics induce in host physiology. In this work, two probiotic formulations with identical species composition, produced in two different sites, the USA and Italy, were utilized to feed *Caenorhabditis elegans*, strains and alterations in lipid composition in the host and bacteria were investigated. Indeed, the multicellular organism *C. elegans* is considered a simple model to study the in vivo effects of probiotics. Nematodes fat metabolism was assessed by gene expression analysis and by mass spectrometry–based lipidomics. Lipid droplet analysis revealed a high accumulation of lipid droplets in worms fed US-made products, correlating with an increased expression of genes involved in the fatty acid synthesis. We also evaluated the lifespan of worms defective in genes involved in the insulin/IGF-1-mediated pathway and monitored the nuclear translocation of DAF-16. These data demonstrated the involvement of the signaling in *C. elegans* responses to the two diets. Lipidomics analysis of the two formulations was also conducted, and the results indicated differences in phosphatidylglycerol (PG) and phosphatidylcholine (PC) contents that, in turn, could influence nematode host physiology. Results demonstrated that different manufacturing processes could influence probiotics and host properties in terms of lipid composition.

## Key points

- *Probiotic formulations impact on Caenorhabditis elegans lipid metabolism;*
- *Lipidomic analysis highlighted phospholipid abundance in the two products;*
- *Phosphocholines and phosphatidylglycerols were analyzed in worms fed the two probiotic formulations.*

**Keywords** Probiotic · *Caenorhabditis elegans* · Lipidomics · Phospholipid

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## Introduction

Probiotics are live microorganisms able to provide health benefits when consumed, generally by improving or restoring the host gut flora (Kechagia et al. 2013). Indeed, probiotic bacteria are available in the form of capsules, drops, powders, or food supplements, such as starter cultures in dairy products. The most common probiotic strains, known for their health-promoting properties, belong to the *Lactobacillus* and *Bifidobacterium* genera (Fijan 2014; Walter 2008). Manufacturing conditions, ingredients, and storage can strongly influence a probiotic product's features, thus affecting its efficacy and safety. In this regard, it has been reported that changes in the growth conditions and the number of culture transfers affect the same strain's properties, influencing the in vivo effects of probiotic bacteria (Grześkowiak et al. 2011). Different manufacturing processes affected the ability of the same probiotic strain to adhere to human epithelial cells and to resist bile salts and acidic pH (Nivoliez et al. 2014; Nivoliez et al. 2012). The model organism *Caenorhabditis elegans* and the broad availability of its mutants can help study the mechanisms and biomolecular pathways involved in host-microorganism interaction.

In *C. elegans*, there is a complex association between animal development, metabolism, and lifespan and several lipid molecules, which are considered crucial for membrane integrity and signaling pathways. Lipids perform different functions, such as forming an impermeable barrier to the external environment, contributing to cell signaling, and providing energy storage (Kimura et al. 2016). The model *C. elegans* permits to study lipid metabolism, thanks to advantages such as visualization of lipid droplets in hypodermic and intestinal cells. Lipid droplets are cytoplasmic organelles surrounded by a monolayer mainly of phosphatidylcholine (PC) and phosphatidylethanolamine, and containing mostly triacylglycerols (TGs) (Witting and Schmitt-Kopplin 2016). Bacterial diet makes nematode a valuable and straightforward tool to study the effects of nutrients.

Moreover, the lipid metabolism-associated genes were identified by comparative genomics (Zhang et al. 2013). Lipidomic technologies become useful to characterize the lipid content of an organism, through the analysis of structure, function, or interaction of cellular lipids, which also play an essential role in nutritional research (Chung et al. 2018). Lipidomics can be applied to different studies on probiotics, among which the metabolic changes that they induce in the human body. In the host's gut, microorganisms interact with each other to produce different metabolites and cause changes in the composition of the organism's lipid composition. The probiotic formulation VSL#3 produced in two different sites, US-made and IT-made, showed properties depending on manufacturing (Palumbo et al. 2018; Trinchieri et al. 2017; Cinque et al. 2017). In this study, the effects of these multispecies probiotic products on lipid metabolism were analyzed on the *C. elegans* model to test the impact due to the interactions of different probiotic species in host cells. After the administration of the probiotics, the lipid composition of *C. elegans* was characterized through lipid droplets staining, phospholipidomics profile, and analysis of expression of genes involved in lipid metabolism. We also investigated the possible mechanisms involved in the observed phenotypes through studies on nematode mutant strains and lipidomic analysis of both probiotic formulations.

## Materials and methods

### Microbial strains and probiotic preparation

VSL#3 is a multispecies probiotic formulation produced in two different sites: Danisco/DuPont (US) and Nutrilinea/CSL (Italy). Commercial packaging reports the following composition: four lactobacillus strains (*L. acidophilus* BA05, *L. plantarum* BP06, *L. paracasei* BP07, *L. debrueckii subsp. bulgaricus* BD08), three bifidobacterium strains (*B. breve* BB02, *B. longum* BL03, *B. infantis* BI04), and *S. thermophilus* BT01, for IT-3; and lactobacilli (*L. acidophilus* DSM 24735, *L. plantarum* DSM 24730, *L. paracasei* DSM 24733, *L. debrueckii subsp. bulgaricus* DSM 24734), bifidobacteria (*B. longum* DSM 24736, *B. breve* DSM 24732, *B. infantis* DSM 24737), and *Streptococcus thermophilus* DSM 24731, for US-7.

In *C. elegans* assays, US-7 and IT-3 were suspended in sterile $H_2O_{dd}$, corresponding to 10 mg of bacterial cells in 25 μL, and then spread on 3.5-cm diameter NGM plates. The standard food *Escherichia coli* OP50 was used as control; it was grown as described in Zanni et al. (2015).

### *C. elegans* strains and growth conditions

The wild-type *C. elegans* strain N2, *daf-2(e1370)* (CB1370) mutants and DAF-16::GFP (TJ356) zIs356 [*daf-16p::daf-16a/b::GFP+rol-6(su1006)*] transgenic strain used in experiments were propagated on nematode growth medium (NGM) spread with US-7, IT-3 or OP50 bacterial lawns. Experiments were performed at 16 °C.

### Lipid droplet visualization

Approximately 60 4-day adult nematodes, fed IT-3, US-7, or OP50, were washed three times in M9 buffer. BODIPY 493/503 (Life technologies) staining was performed as described by (Schifano et al. 2019a, b). Subsequently, worms were mounted onto 3% agarose pads containing 20 mM sodium azide and observed with a Zeiss Axiovert 25 microscope at × 10X and × 32 magnifications. Median fluorescence intensity was measured with the ImageJ software, measuring the ratio

of pixels per area of the worm. For each sample, ten transgenic nematodes were analyzed, and the mean value was reported. The experiment was repeated three times.

## Lipid extraction procedure

Phospholipid extraction was performed on 4-day adult *C. elegans* propagated on NGM plates spread with US-7, IT-3, or OP50 bacterial lawns. Each *C. elegans* pellet was subjected to homogenization (Ultra-Turraz homogenizer IKA LaborTechinik), and sonication (five cycles of 10 s each, amplitude 70%) by using a probe sonicator (IKA LaborTechnik). Samples were then centrifuged (15 min 17,100 rcf), and 150 μL of supernatant for each sample was undergone to extraction procedure, as already reported in our previous work (Del Boccio et al. 2017). Briefly, after vortexing, 300 μL of methanol was added for protein precipitation. The obtained solution was vortexed, mixed for 5 min at 10 °C, and centrifuged (15 min, 10 °C, 17100 rcf). After centrifugation, 1 mL of methyl *tert*-butyl ether (MTBE) and 250 μL of water were added to the supernatant (350 μL), vortexed, mixed, and centrifuged at the same conditions previously described. The upper MTBE phase was collected, dried, and finally dissolved in 100 μL of acetonitrile (ACN), vortexed, and centrifuged. A total of 90 μL of the supernatant solution was recovered, and 40 μL was transferred in the vials for LC-MS/MS analysis. The remaining 50 μL was stored at − 80 °C for further investigations.

The same extraction procedure was also performed on VSL#3 multispecies probiotic formulations: US-7 and IT-3. The powder was resuspended in PBS (Dulbecco A, Thermo Scientific) to obtain a final concentration of 0.2 g/mL. After centrifuging (10 min, 3220 rcf), the pellets were resuspended in 2 mL of PBS and subjected to three freezing/thawing cycles and sonication (five cycles of 10 s each, amplitude 70%). The samples were centrifuged (15 min, 3220 rcf), and 200 μL of supernatant was subjected to lipid extraction procedure, as already described.

## Lipidomic profiling by LC-MS/MS

The LC-MS/MS method for lipidomic profiling was performed as already described in our previous work (Del Boccio et al. 2017).

The lipid extract was injected (20 μL) and separated by Alliance HT 2795 HPLC Waters Corporation). The separation was performed through an Atlantis HILIC silica 3 μm, 150 mm × 2.1 mm (Waters Corporation) column, using a gradient of formic acid 0.1% (solvent A) and acetonitrile (solvent B) as follow: 92% B for 5 min; to 70% B in 15 min; isocratic 70% B for 2 min; to 35% B in 22 min; finally, re-equilibration in 27 min. The elution flow was set to 200 μL/min. The LC system was coupled on-line with a triple quadrupole (Quattro Ultima Platinum Micromass, Waters Corporation) through an ESI source operating in positive ion mode. A 3.5 kV tension was applied on the capillary, while a 60 V tension was applied on the cone. MS/MS fragmentation functions were used to obtain the profile of biological phospholipids. Argon was used as the collision gas. The method allows an open profile of the following classes of phospholipids: phosphatidylcholines (PC), sphingomyelins (SM), phophatidylethanolamines (PE), phosphatidylserines (PE), phosphatidylglycerols (PG). The profile of biological phospholipids was performed by MS/MS fragmentation functions performed by argon as the collision gas. For the detection of several classes in a single analysis, data acquisition was achieved through four different MS/MS functions, as shown in Table 1.

## Lipidomics data processing and statistics

Lipidomic data were processed using the MarkerLynx software (Waters, Milford, MA, USA), allowing deconvolution, alignment, and data reduction to give a table of mass and the relative retention time pairs with relative intensities for all the detected peaks. The minimum intensity considered (expressed as % BPI) was set to 10%. The lipidomic data matrix obtained by MarkerLynx was used for partial least squares discriminant analysis (PLS-DA) using SIMCA-P+. VIP (Variable Importance in the Projection) was employed to reflect the variable importance in the discriminant analysis. The major discriminant variables were selected and underwent the Student's *t* test or two-way ANOVA test using GraphPad Prism (GraphPad Software, Inc. USA). The significantly different variables were tentatively identified by Lipidmaps Database and Human Metabolome Database (HMDB) and by using the MS/MS data and the retention time. Heatmap and Volcano Plot were performed by Metaboanalyst 3.0 without any pre-processing manipulation.

**Table 1** MS/MS fragmentation functions performed for the detection of the subclasses of phospholipids investigated

| Phospholipid class (parent) | MS/MS function | Collision energy (eV) | Acquisition time (min) |
|---|---|---|---|
| PG | Neutral loss of 172 m/z | 15 | 2–15 |
| PE | Neutral loss of 141 m/z | 15 | 7–14 |
| PS | Neutral loss of 185 m/z | 20 | 10–20 |
| PC, SM | Parent scan of 184 m/z | 25 | 10–21 |

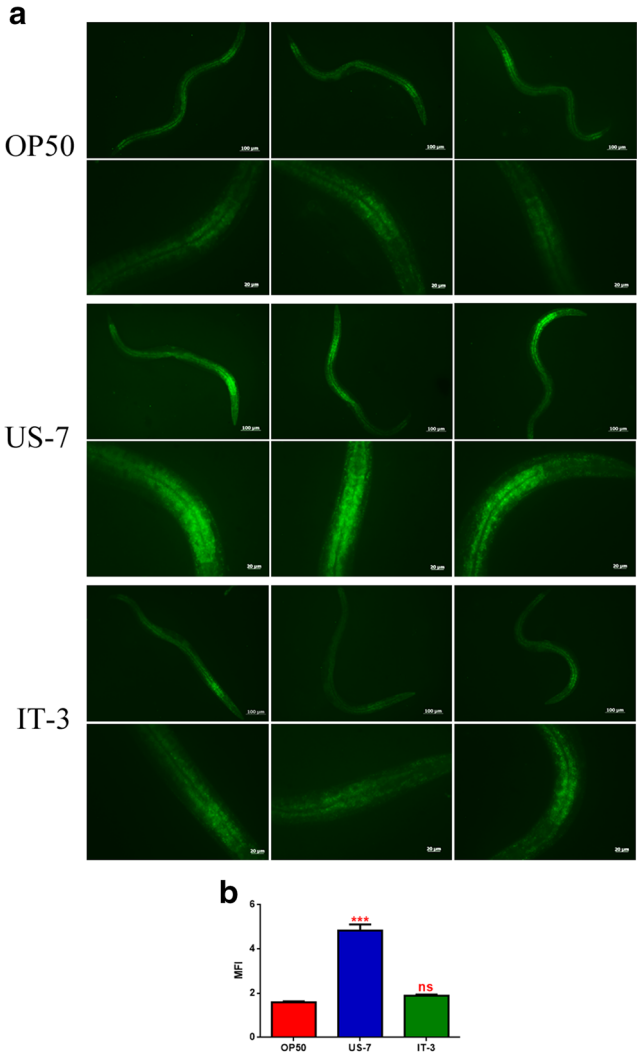



**Fig. 1** Visualization of lipid droplets in *C. elegans*. **a** BODIPY™ 493/503 staining of lipid droplets in 4-day adult worms fed with OP50, US-7, or IT-3 products. **b** Median fluorescence intensity of worm strained with BODIPY. Statistical analysis was evaluated by one-way ANOVA with the Bonferroni post-test; asterisks indicate significant differences (ns: not significant; ***$p < 0.001$). Bars represent the mean of three independent experiments with $n = 30$

## RT-qPCR

At the stage of 4 days of adulthood, total RNA from 200 worms for each sample was isolated with RNeasy midi kit (Qiagen) according to manufacturer's instructions and then digested with 2 U/μL DNAse I (Ambion). One microgram of each sample was reverse-transcribed using oligo-dT and enhanced Avian reverse transcriptase (SIGMA, Cat. Number A4464), as described in (Zanni et al. 2015). s*bp-1*, *pmt-1*, *fat-7*, *acs-2*, and *sams-1* mRNA levels were analyzed. The differences between the mean CT value of each sample and the CT value of the housekeeping gene (*act-1*) were calculated. Primers used in this study are reported in Table S1. The experiment was performed in triplicate.

**Fig. 2** LC-MS/MS lipidomic analysis of ***C. elegans***. **a** Scores scatter plot calculated on two components by using 40 phospholipid species identified in *C. elegans* grown up with a standard diet (OP50, as red squares), diet based on US-7 (blue dots), or IT-3 (green diamonds). The Partial Last Square Discriminant Analysis (PLS-DA) was used to classify the three groups examined, obtained by R2Y = 0.72 and Q2(cum) = 0.37. **b** Heatmap showing the abundance of the 22 phospholipids signals identified as variable important for the projection (VIP > 1). Lipid levels are indicated by a color code: high (red) and low (green). The yellow box includes the lipid species resulted differential at the univariate Student's *t* test between the *C. elegans* grown up with US-7 and IT-3 diets (PC 38:7, PC 32:0, PC 31:0, PC 37:5, and PC 34:1). Such lipid species were reported in the histograms in panel c, as mean ± SD of their relative intensity (*$p < 0.05$)

## Fluorescence analysis of *C. elegans* DAF-16::GFP strain

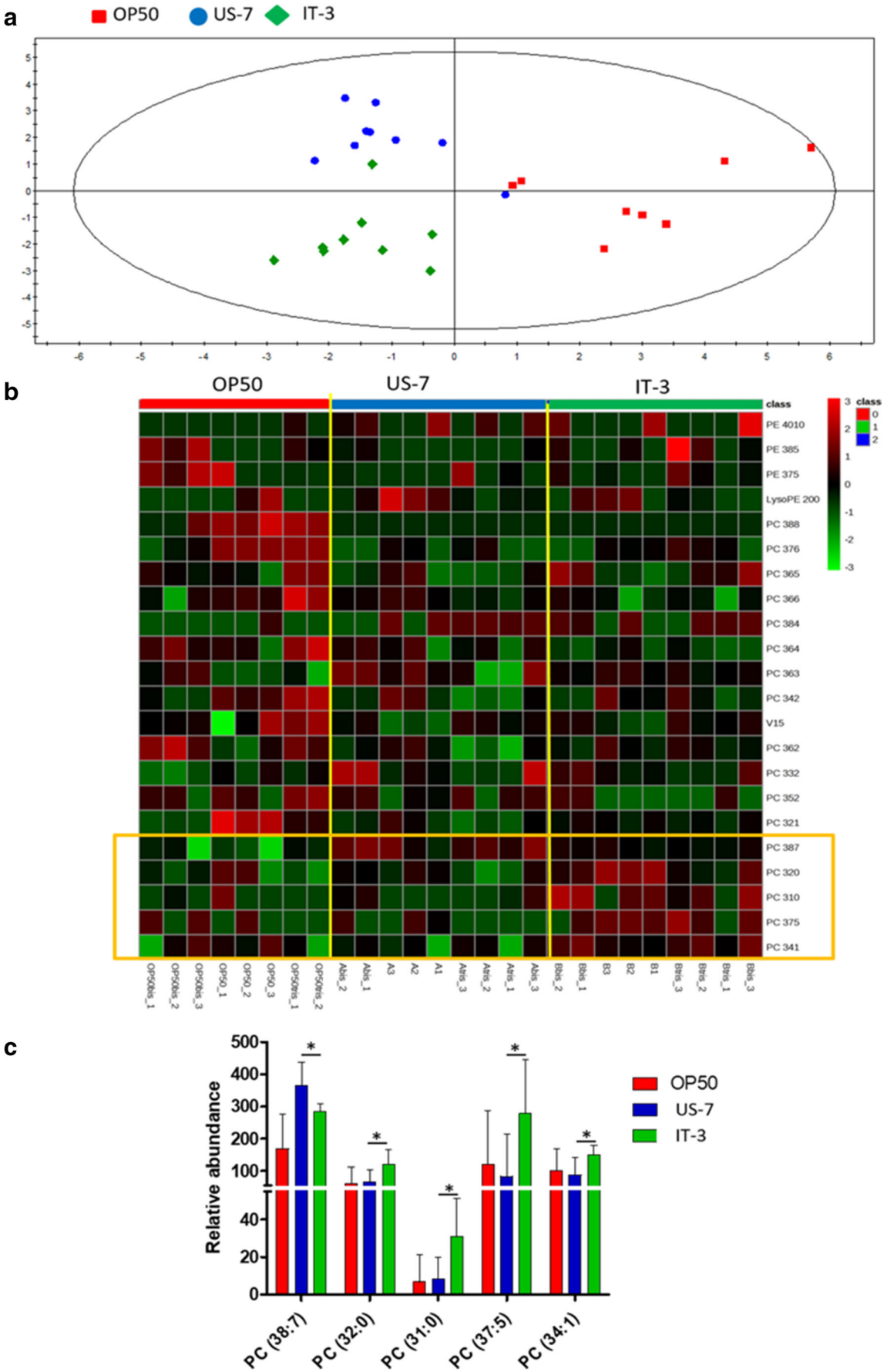
Synchronized transgenic worms were transferred daily to NGM plates containing two different products and were grown at 16 °C, as described above. At the stage of 4 days, the adult worms were put at 37 °C for 30 min to induce stress. Then, they were anesthetized, and the nuclear translocation was observed according to Schifano et al. (2019a, b). For each sample, 10 worms at the stage of 4 days of adulthood were observed with a Zeiss Axiovert 25 microscope at × 10 magnification. Quantification of fluorescence intensity was evaluated with the ImageJ 1.43 (NIH) software measuring the percentage of GFP-positive nuclei in transgenic worms.

## Lifespan assays

Wild-type N2 and *daf-2* mutant adults were allowed to lay embryos for 8 h directly on NGM, seeded with the lawns made up of the two probiotic formulations or the OP50, as indicated. Adults were then sacrificed, and lifespan assays started when the progeny became fertile and monitored daily, as described in Zanni et al. (2017) and Amrit et al. (2014). All the lifespan were performed in triplicate at 16 °C. In each experiment, plates with 60 worms were prepared in triplicate.

## Statistical analysis for *C. elegans* experiments

The statistical analysis was performed by Student's *t* test or one-way ANOVA analysis coupled with a Bonferroni post-test (GraphPad Prism 5.0 software, GraphPad Software Inc., La Jolla, CA, USA). For lifespan experiments, Kaplan-Meier survival plot analysis was performed. Differences with *p* values < 0.05 were considered significant and were indicated as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ns: not significant. Experiments were performed at least in triplicate.


a
OP50
US-7
IT-3
b
OP50
US-7
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class
PE 4010
PE 385
PE 375
LysoPE 200
PC 388
PC 376
PC 365
PC 366
PC 384
PC 364
PC 363
PC 342
V15
PC 362
PC 332
PC 352
PC 321
PC 387
PC 320
PC 310
PC 375
PC 341
c
Relative abundance
OP50
US-7
IT-3
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PC (32:0)
PC (31:0)
PC (37:5)
PC (34:1)

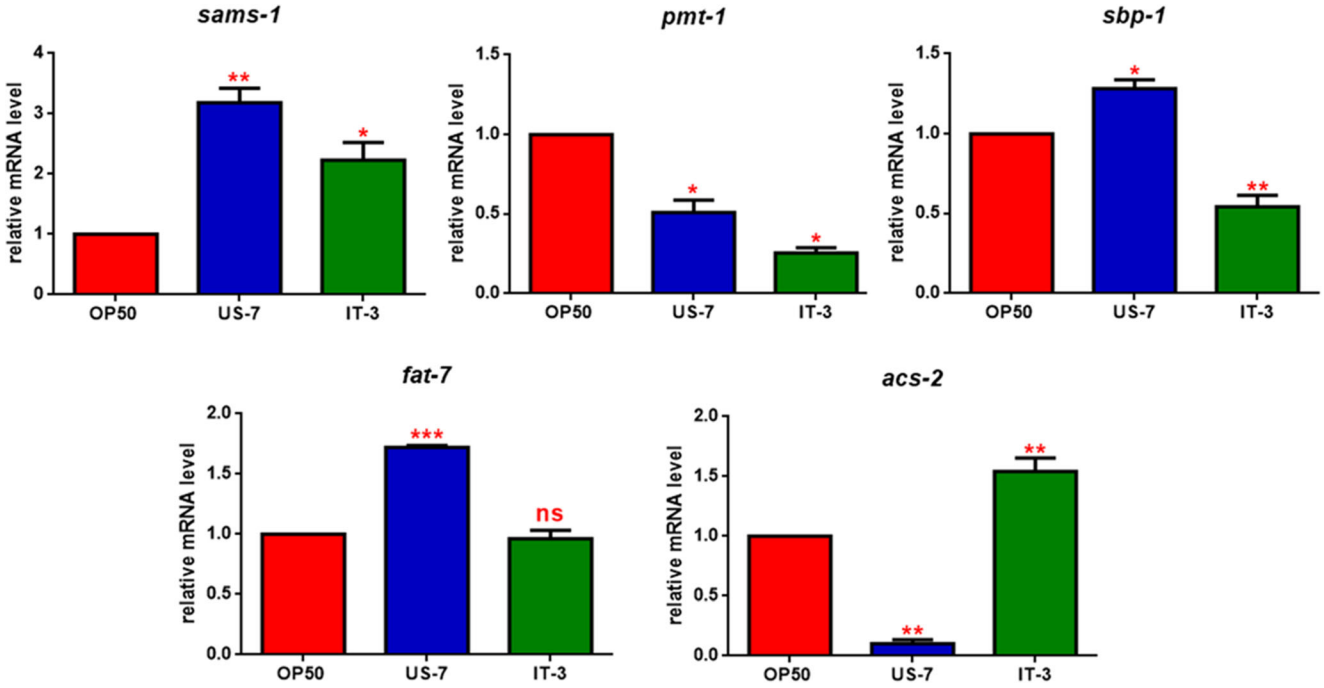



**Fig. 3** RT-qPCR analysis of lipid metabolism genes. Expression of *sams-1*, *sbp-1*, *pmt-1*, *fat-7*, and *acs-2* genes in US-7-, IT-3-, or OP50-fed animals at the stage of 4 days of adulthood. Histograms show the expression of genes involved in lipid metabolism detected by real-time PCR. Experiments were performed in triplicate. Data are presented as mean ± SD (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ns: not significant)

## Results

### US-7 and IT-3 products differently affect lipid droplets and lipidomic profile in *C. elegans*

Diet affects lipid metabolism in several organisms, including *C. elegans* (Brooks et al. 2009). To evaluate the impact of two probiotic formulations on fat storage in nematodes, lipid droplet accumulation was assessed. When nematodes fed US-7 product, an increase in fat storage was observed in 4-day adult worms after BODIPY™ 493/503 staining, compared with OP50-fed individuals used as control. By contrast, after the administration of IT-3 formulation, wild-type worms showed an accumulation of lipid droplets similar to that observed in OP50-fed nematodes (Fig. 1).

We also studied the main classes of phospholipids (see Table 1) by the LC-MS/MS lipidomic analysis performed on *C. elegans* subjected to the different diets, obtaining hundreds of phospholipids molecular species for each analyzed sample. To highlight differential lipid profiling, data were processed through a multivariate statistical analysis. After data matrix elaboration, 40 PCs and PE species were used to reclassify the *C. elegans* worms subject to the three different types of diets through the Partial Least Square Discriminant Analysis (PLS-DA). The multivariate analysis shows an unambiguous separation between the three groups (OP50 by red square, US-made are indicated by blue dots, and IT-made by green diamonds), thus obtaining a PLS-DA predictive model able to reclassify the study groups (R2Y = 0.72 and Q2(cum) = 0.37), as shown in Fig. 2a. Discriminant phospholipids identified as

**Table 2** Lifespan analysis of wild-type N2 and *daf-2* mutant worms. Kaplan-Meier survival plot of N2 and *daf-2* mutant worms fed US-7 and IT-3 formulations. The lifespan of OP50-fed animals was reported as control. Three experiments were performed in triplicate for each condition

| *C. elegans* strain | Diet | *n* | Median lifespan | Maximum lifespan | Number of censored | Statistics |
|---|---|---|---|---|---|---|
| N2 | OP50 | 540 | 26 ± 1.3 | 33 ± 1.2 | 18 | - |
| N2 | US-7 | 540 | 36 ± 0.9 | 41 ± 1.0 | 17 | $p < 0.01$ versus OP50 fed-N2 |
| N2 | IT-3 | 540 | 32 ± 0.8 | 39 ± 0.9 | 19 | $p < 0.05$ versus OP50 fed- *daf-2* |
| *daf-2* | OP50 | 540 | 45 ± 0.9 | 51 ± 0.7 | 21 | - |
| *daf-2* | US-7 | 540 | 42 ± 1.3 | 49 ± 1.2 | 16 | ns versus OP50 fed- *daf-2* |
| *daf-2* | IT-3 | 540 | 43 ± 1.4 | 49 ± 1.3 | 20 | ns versus OP50 fed- *daf-2* |

*ns* not significant

variable important for the projection (VIP > 1) were confirmed through a univariate test. All the lipid signals, identified as significant, were depicted in Fig. 2b, as a heatmap. The heatmap provided an overview of the lipids signals and their abundance, in terms of overexpression (in red) or underexpression (in green), in *C. elegans* animals grown up with OP50, US-7, and IT-3 diets. Moreover, confirmed differential lipids in *C. elegans* species grown up with IT-3 diet, compared with the US-7 diet, were reported in the histograms in Fig. 2c. In particular, we found significantly higher levels of PC (32:0), PC (31:0), PC (37:5), and PC (34:1), and lower levels of PC (38:7).

## US-7 and IT-3 products differently influence lipid metabolism-associated gene expression in *C. elegans*

To identify the genes possibly responsible for the differences in fat storage induced by the two formulations, a real-time qPCR analysis was performed for *sams-1*, *sbp-1*, *pmt-1*, *fat-7*, and *acs-2* genes, whose involvement in lipid metabolism have been described (Watts and Ristow 2017; Walker et al. 2011). The results showed that the diet based on US-7 induced an increased transcription of genes related to the synthesis of lipids, such as one of the three Δ9 desaturases (*fat-7*), the homolog of the mammalian transcription factor SREBP-1c (*sbp-1*), which facilitates fat storage in mammals and the *S*-adenosylmethionine synthetase (*sams-1*), relevant for the synthesis of PC (Fig. 3).

The expression of *pmt-1*, also involved in PC synthesis, and *acs-2*, required for the mitochondrial β-oxidation, was instead reduced when compared with that observed in the control animals. By contrast, in worms fed IT-3, an upregulation of *acs-2* was found, while the other transcripts were reduced with respect to those of US-7 fed animals (Fig. 3).

## The effect of US-7 and IT-3 on *C. elegans* lifespan is DAF-2-dependent

Since both probiotic formulations induced an increased lifespan in wild-type animals compared with the control (Table 2), to further investigate the involvement of insulin/IGF-1 signaling (IIS) pathway, the survival rate of DAF-2 mutants was examined. Indeed, nematode median survival was recorded at days 32 and 33 when worms fed IT-3 and US-7, respectively, as compared with day 26 in the case of OP50-fed worms.

In long-lived DAF-2 mutants, localization of DAF-16 to the nucleus induces the expression of genes involved in increased lifespan, fat metabolism, stress responses, and immunity (Murphy et al. 2003). In particular, these mutants exhibit a higher de novo fat synthesis than wild-type worms (Perez and Van Gilst 2008).

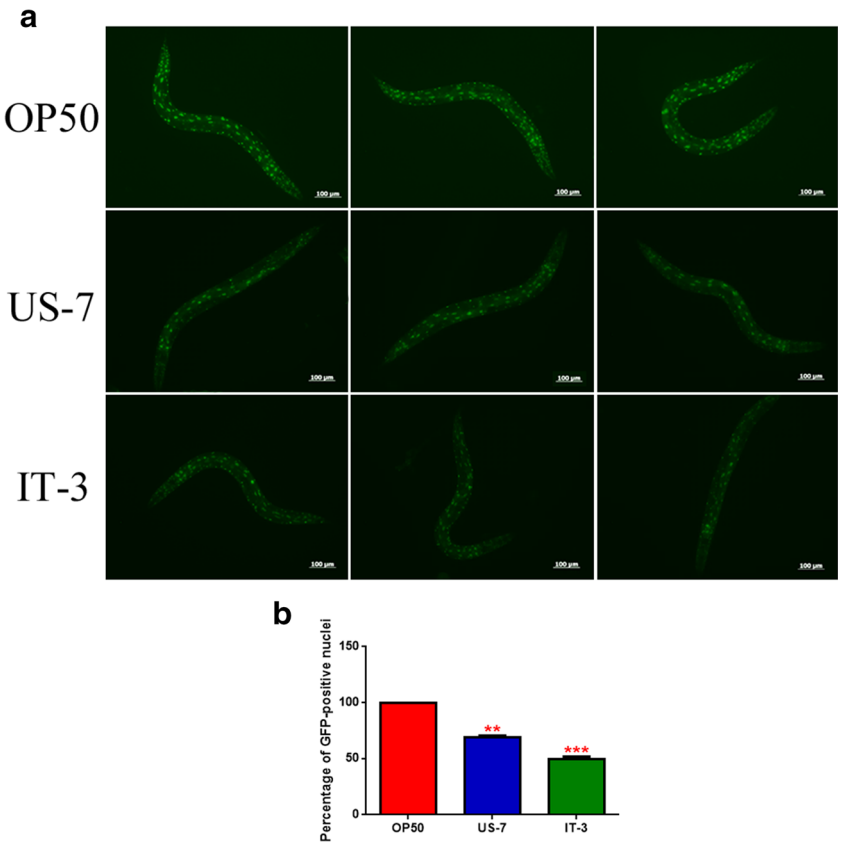



**Fig. 4** Visualization of DAF-16 translocation in *C. elegans* nuclei. **a** Analysis of the translocation of DAF-16 in the nucleus in the transgenic DAF-16::GFP strain. Fluorescence microscopy of worms at the stage of 4-day adult fed with OP50, US-7, or IT-3. Scale bar = 100 μm. **b** Percentage of GFP-positive nuclei. Statistical analysis was evaluated by one-way ANOVA with the Bonferroni post-test; asterisks indicate significant differences (**$p < 0.01$; ***$p < 0.001$). Bars represent the mean of three independent experiments with $n = 20$

The median lifespan of mutants fed US-7 or IT-3 products from embryo hatching resulted in being not significantly different as compared with OP50-fed mutant worms (Table 2). In particular, 50% of worm viability after IT-3 and US-7 product administration was recorded respectively on days 43 and 42 compared with day 45 recorded in OP50-fed animals. Therefore, the effect of the two probiotic products on *C. elegans* lifespan was dependent on the activation of DAF-2.

### US-7 and IT-3 products affect DAF-16 nuclear translocation in transgenic DAF-16::GFP *C. elegans*

Increased accumulation of lipid droplets has been shown to correlate with the insulin/IGF-1-signaling (IIS) pathway (Horikawa and Sakamoto 2010). In particular, the activation of the transcription factor DAF-16 regulates fat metabolism in *C. elegans* (Shi et al. 2013). Therefore, the effect of the two probiotic formulations on nuclear translocation of DAF-16 was analyzed in the transgenic DAF-16::GFP worm strain at the stage of 4 days of adulthood. To induce stress in worms, a heat stress impulse was given, putting plates at 37 °C for 30 min. Microscopy analysis revealed that the translocation of DAF-16 in IT-3-fed worms was notably lower than that of US-7 formulation-fed animals. In general, a reduction of about 30% and 50% of the GFP translocation in the nucleus was observed when worms fed US-7 or IT-3 products compared with that of control (Fig. 4).

### US-7 and IT-3 products show different lipidomic profile

To analyze the lipid composition of the two formulations, the lipidomic investigation on US-7 and IT-3 has been performed. Lipidomic data were undergone to statistical analysis, visualized as volcano plot (Fig. 5a). The lipidomic profile resulted statistically different, as reported in histograms in Fig. 5b. We observed that five PC species out of seven resulted more abundant in probiotic formulation IT-3, and all the PG species resulted more abundant in probiotic formulation US-7.

## Discussion

The relations between the intestine, the microbial flora, and lipids remain mostly unexplored. It is not just a matter of calories and changes in body weight, as it is known that lipids can behave as messengers freely diffusing through membranes and binding to protein receptors, which mediate the effects of these lipids on specific cellular responses.

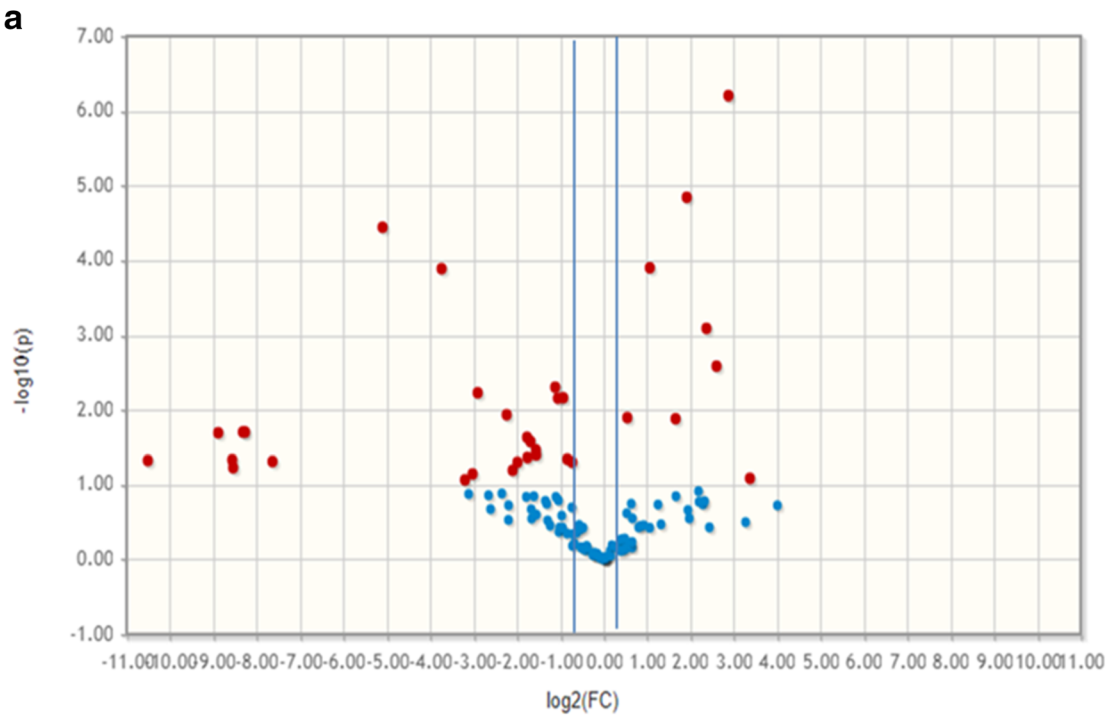
Probiotics are useful in a wide range of clinical conditions as liver diseases, inflammatory bowel diseases, allergy, and recently, even in some neurological conditions (Liu et al. 2018). However, different industrial processing and matrices may easily result in a loss of the original characteristics of the bacterial strain(s) by modifying the strains' life-cycle and gene expression (Bianchi et al. 2020). Changes in the manufacturing of a probiotic may also affect the lipid machinery of the bacterial cell, which could affect the lipid signaling system.

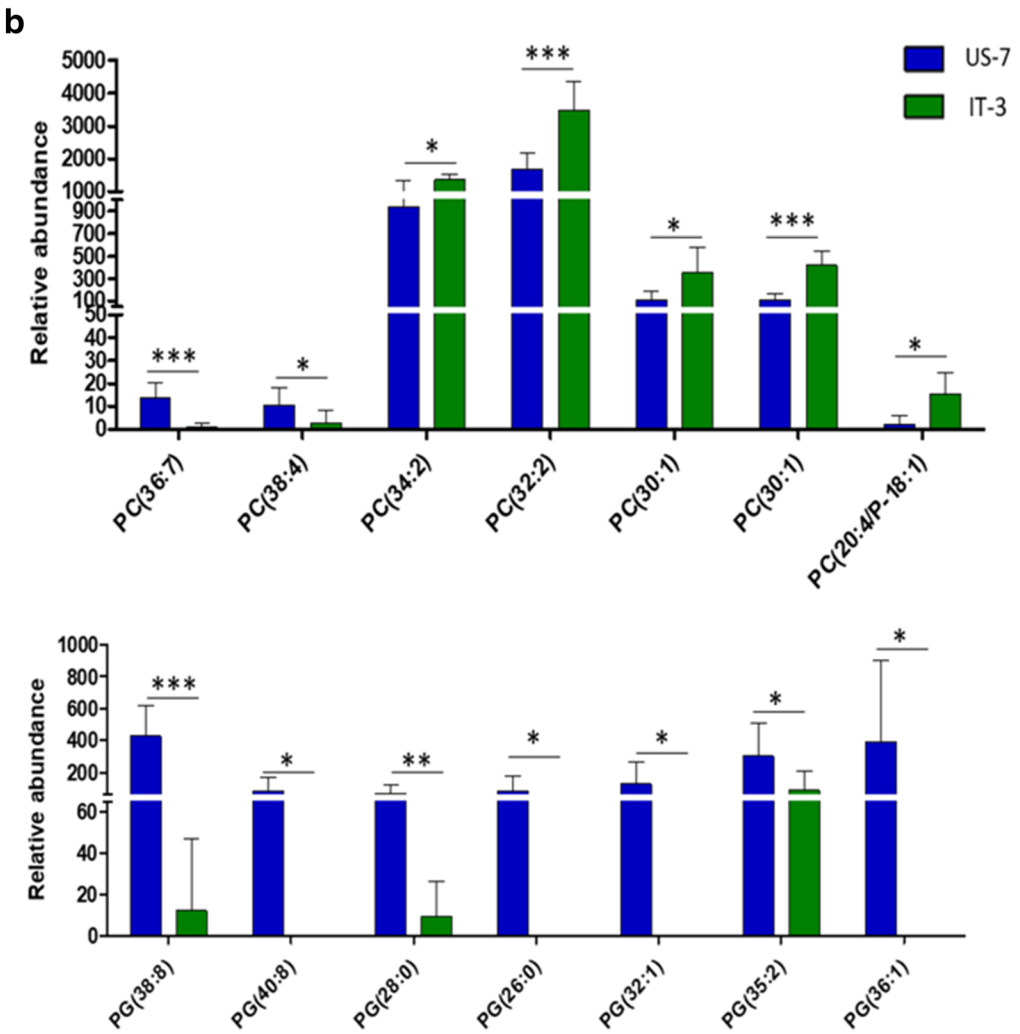
Genomic and most recently, metabolomic studies revealed differences between the two VSL#3 products (Douillard et al. 2018; Biagioli et al. 2017). This probiotic formulation was used to assess *C. elegans* as an in vivo tool to analyze manufacturing changes at the lipid level. Typically, worms can synthesize different lipid species. Sphingolipids, fatty acids, glycerophospholipids, or sterol lipids play many essential roles (Watts and Ristow 2017). In nematodes, lipids are mainly stored as triacylglycerols (TAGs) in droplets surrounding by a monolayer of the phospholipids PC and PE. These droplets are localized in gut granules and hypodermal cells and regulated by different players, among which the insulin/insulin-like growth factor 1 receptor homolog DAF-2 (Ashrafi 2007; Ashrafi et al. 2003). DAF-2 receptor activation is involved in many processes, affecting not only lipid metabolism but also lifespan, reproduction, and starvation (Kaletsky and Murphy 2010; Gami and Wolkow 2006).

Here, lipidomics investigation on *C. elegans* demonstrated that phospholipid profile in vivo could be modulated according to the probiotic diet. In particular, our lipidomic data, obtained through the analysis of *C. elegans* fed with US-7 probiotic compared with IT-3 product, showed differential PC profile and almost all the most significant differential PCs (PC 32:0, PC 31:0, PC 37:5, and PC 34:1) have resulted decreased in *C. elegans* animals fed with US-7 probiotic.

These data might seem to be in contrast to the increased fat storage found in worms fed with US-7 compared with those supplied with OP50 standard diet and IT-3. However, phospholipids, particularly PCs, PEs, and SMs are the principal components of both the droplet monolayer and the cellular membrane. Therefore, the differential PC species found significantly decreased in worms fed US-7 cannot be directly correlated with the enhanced lipid droplets in those animals. At the same time, most likely, the probiotic formulation might, in turn, affect lipid metabolism in vivo.

**Fig. 5** LC-MS/MS lipidomic analysis of probiotic formulation IT-3 and US-7. **a** Statistical analysis represented as the Volcano Plot, a combination of fold change (FC) and *t* tests. The *x* axis is log2 FC, and the *y* axis is log10 (*p* value) obtained by non-parametric test. FC values referred to IT-3 versus US-7. The red dots were significant with the − log10 (*p* value) > 1. Blue dots are ns. The red dots on the right represent the lipids with higher levels in probiotic formulation IT-3, while the dots on the left are the lipids with lower levels in probiotic formulation IT-3. **b** Relative abundance (based on the total ion count) of the lipid species identified as significantly different in the comparison (divided into 7 PCs, and 7 PGs). Bars in the histograms were represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ at the Student's *t* test in the comparison between IT-3 and US-7 formulations


a
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-log10(p)
-11.00 10.00 9.00 8.00 7.00 6.00 5.00 4.00 3.00 2.00 1.00 0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00
log2(FC)


b
US-7
IT-3
Relative abundance
5000
4000
3000
2000
1000
900
700
500
300
100
50
40
30
20
10
0
***
*
***
*
*
***
*
PC(36:7)
PC(38:4)
PC(34:2)
PC(32:2)
PC(30:1)
PC(30:1)
PC(20:4/P-18:1)
Relative abundance
1000
800
600
400
200
60
40
20
0
***
*
**
*
*
*
*
PG(38:8)
PG(40:8)
PG(28:0)
PG(26:0)
PG(32:1)
PG(35:2)
PG(36:1)

Actually, in worms fed with US-7 and IT-3 diet, a different expression of genes involved in lipid metabolism was observed. *Pmt-1* gene, directly involved in PC biosynthesis, resulted reduced in worms after administration of the two probiotic products, compared with OP50-fed nematodes. It has been reported that the decrease of PC biosynthesis led to the transcriptional factor SBP-1 activation, stimulating the synthesis of unsaturated fatty acids mediated by Δ9 desaturases *fat-7* (Pilon and Svensk 2013). In US-7-fed worms, an increase of expression of *sbp-1* and *fat-7* transcripts was observed, confirming the possible involvement of this pathway in the accumulation of lipid droplets. Instead, in worms fed IT-3, lower lipid droplets correlated with a reduced expression of *sbp-1* and *fat-7*.

Moreover, RT-PCR analysis revealed a substantial decrease in the expression of *acs-2* in worms fed US-7 product, while IT-3-fed worms showed higher levels of *acs-2* transcripts. It has been described that acyl-CoA synthetase *acs-2* activation links with a reduction of desaturase expression in *C. elegans* (Nomura et al. 2010). In particular, ACS-2 is a key crucial enzyme of the β-oxidation pathway, activating fatty acids for β-oxidation (Coleman et al. 2002). Therefore, the accumulation of fat storage in US-7-fed nematodes was probably due to an imbalance between synthesis and β-oxidation of fatty acids. Lastly, nematodes fed the two probiotic formulations showed a higher expression of *sams-1* transcripts than OP50. *Sams-1* encodes *the S*-adenosyl methionine synthetase 1, a conserved enzyme regulating lipid droplet size and homeostasis between PC synthesis and lipolysis (Ehmke et al. 2014). Indeed, downregulation of *sams-1* or *pmt-1* causes a TAG accumulation and a decrease in PC (Li et al. 2011). Recent works have reported the involvement of *sams-1* in *C. elegans* aging and longevity (Liu et al. 2019; Cabreiro et al. 2013), so the increased *sams-1* transcription in worms fed US-7 and IT-3 could be related to pro-longevity effects observed in wild-type worms. The reduction of PCs, the enhanced expression of *sams-1*, *sbp-1*, and *fat-7*, and the downregulation of *acs-2* strengthen the evidence of a modulation of lipid metabolism after administration probiotics, which could be related to pro-longevity effects. When DAF-2 pathway is turned off, the transcription factor DAF-16, after phosphorylation, translocates in the nucleus influencing the expression of genes involved in longevity, stress responses, lipid synthesis, and other signals (Roselli et al. 2019; Henderson and Johnson 2001; Ogg et al. 1997). *daf-2* mutant worms, carrying a mutation in the receptor of this cascade, show increased longevity and a higher amount of lipid droplets (Shi et al. 2013; Kenyon et al. 1993). In *daf-2* mutants fed with the two probiotic products, the lifespan resulted not significantly different as compared with control, demonstrating that the effects exerted on *C. elegans* lifespan could be dependent on DAF-2 activation. On the contrary, analysis of transgenic DAF-16::GFP strain highlighted a reduced translocation of the transcriptional factor in the nucleus, confirming an alteration of this cascade in *C. elegans* response to probiotic administration. It was reported that increased DAF-16 nuclear localization is associated with increased lifespan and stress resistance (Roselli et al. 2019; Henderson and Johnson 2001; Ogg et al. 1997; Kenyon et al. 1993). In this case, decreasing of DAF-16 nuclear localization in wild-type worms fed with US-7 and IT-3 might be due to the involvement of other molecular players, such as *skn-1* and *hsf-1*, which are known to regulate oxidative stress responses and longevity (Koch et al. 2014; Baumeister et al. 2006). It is reported that host–microbiota interaction affects immunity, fat metabolism, drug metabolism, and aging. It is known that, among various mechanisms of action, probiotics are believed to exert their effects by gut colonization, production of antimicrobial substances, and enhancement of host mucosal barrier integrity and immune modulation (Kumar et al. 2019). Therefore, lipid droplet accumulation phenotypes, longevity, and DAF-16 nuclear accumulation could be due to the ability of the two formulations to colonize *C. elegans* gut and interact with hosts cells, activating different pathways. Finally, to confirm the influence of the probiotic diet in the lipid metabolism of *C. elegans* animals, for the first time lipidomic fingerprint of a probiotic formulation produced in two different sites was performed. The VSL#3 product produced in the USA was more abundant in PG species than the VSL#3 IT-made. These differences might be responsible for the different effects of US-7 and IT-3 on fat metabolism and different lipid droplet accumulation observed in *C. elegans* animals. In conclusion, we evaluated the relationships between probiotic bacteria and alterations in the *C. elegans*' lipid composition in this study, confirming that different manufacturing processes induce different lipidic modifications and biological activities.

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**Author contributions** DU designed the *C. elegans* experiments and wrote the paper.

ES performed *C. elegans* experiments and wrote *C. elegans* results.

IC executed formal lipidomics data analysis and wrote lipidomics results.

DP edited lipidomics results and data processing and PDB performed conceptualization of lipidomics experiments. DU, HJH, and PDB critically revised and edited the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics approval** Not applicable

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