PATHWAY-27 specific objectives can be summarized as follow:

- critical evaluation of the bioactive-food matrix interaction;
- evaluation of the modifications occurring during food processing and storage;
- address possible synergies between different bioactives;
- investigate on digestibility of BEF and consequent bioavailability of bioactives;
- investigate on BEF effectiveness using strong clinical markers;
- evidence new markers of effectiveness by means of omics techniques:
- develop an integrated *in vitro-in vivo* approach allowing a better understanding of the clinical effect of BEF and its underlying mechanism/s of action.

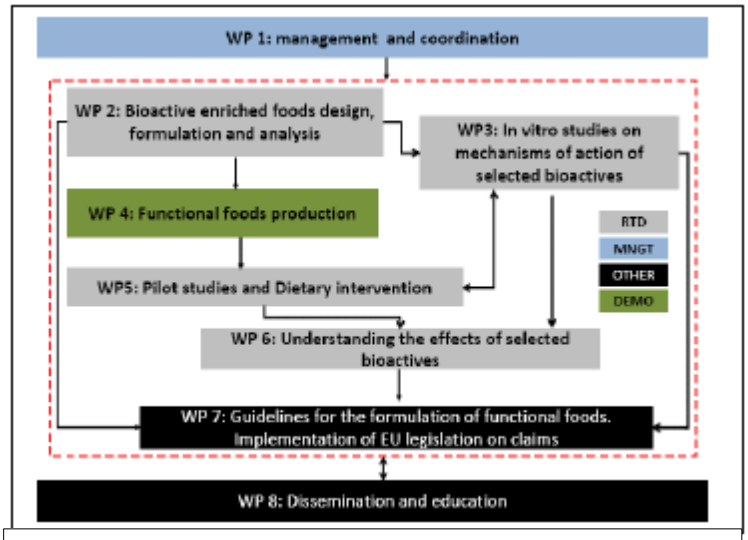

Figure 1. PATHWAY-27 PERT diagram

Highlights

- Three matrices were chosen based on their frequency of consumption in the diet, and three food within each matrix was enriched with the bioactives alone and in combination. In total, 45 BEF prototypes were developed.
- The ingredients containing high enough concentration of the different bioactive compounds (AC, OBG and DHA) were produced, taking care of their safety.
- After a pre-selection based on organoleptic characteristics, 30 BEF remained in the focus.
- A complete sensory characterization of the 30 selected BEF was done by the trained sensory panel. Consumer tests were also performed.
- A questionnaire to assess food product liking was developed. The questionnaire was used within the PATHWAY-27 human intervention studies, and could be useful in other clinical studies administering food.
- Characterization of BEF by NMR spectroscopy allowed a better understanding of the bioactive/food matrix interactions.
- Bioactive compound bioaccessibility was assessed by *in vitro* digestion.
- A methodology based on the integrated analysis of BEF characteristics was developed to select the best 3 products (one in each matrix) to be used in intervention studies.
- The bioactive content of the selected BEF was determined after long storage time, considering this parameter as an additional one to evaluate the product shelf-life.
- The three selected products (milkshakes, biscuits, pancakes) enriched with five different bioactive combinations were produced for use in pilot studies.

- The safety characteristics of BEF were determined and certified, and their shelf-life evaluated also considering bioactive retention.
- The bioactive content in each production batch was assessed to ensure reproducibility of tested food, which is one of the basic requirements of EFSA for the substantiation of the health claims.

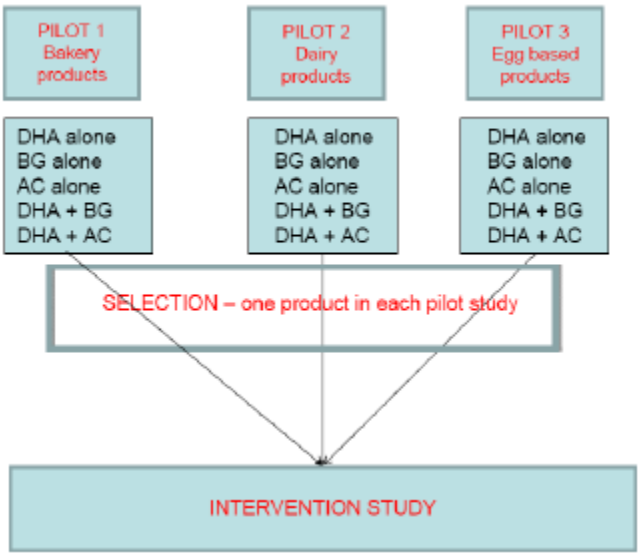

Figure 2. Scheme of pilot studies

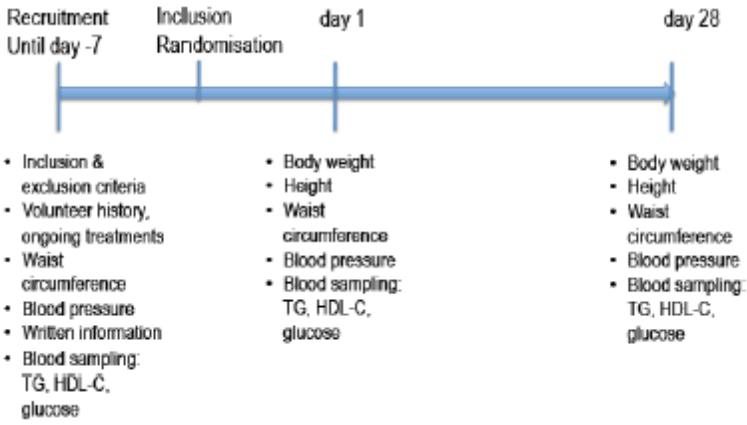

Figure 3. Pilot study protocol

Table 1. MetS diagnosis criteria

- elevated waist circumference (men ≥ 102 cm; women ≥ 88 cm)
- elevated fasting triglycerides (≥ 150 mg/dL)
- reduced fasting HDL-cholesterol (men ≤ 40 mg/dL; women ≤ 50 mg/dL)
- elevated blood pressure (systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg) or hypotensive treatment

- elevated fasting glucose (≥ 110 mg/dL)

Food	Selected enrichment
	DHA+ AC
	DHA+ AC
	DHA+ OBG

Figure 4: BEF selected in pilot studies

Highlights

- Pilot studies allowed selecting the most promising BEF in each matrix.
- The three BEF selected in the pilot studies and the corresponding placebos were produced for use in the LIS.
- A strategy was developed to ensure an un-stoppable supply of fresh BEFs and placebos to recruiting centres during pilot studies and the LIS.
- The foodomics approach was applied to assess the effect of the incorporation of a functional ingredient on the reproducibility and/or stability of three different food matrices.
- Since the LIS needed to be extended, a strategy was developed to minimise food waste and the production efforts ensuring the blindness in the study.
- Production issues, critical control points, management and problem-solving activities for the industrial exploitation were carefully addressed.

Highlights

- A four-arms large intervention study (LIS) was designed and performed. The LIS was a multicentre, randomized, placebo-controlled, parallel-arm dietary intervention study. Intervention lasted 12 weeks.
- Volunteers at risk for or affected by MetS were enrolled in the study. Modification in triglycerides (TG) and HDL-cholesterol blood levels were the primary endpoints.
- 5401 putative volunteers were contacted by the 4 RCs, of whom 1361 accepted to carry out the screening visit. 420 resulted eligible and 325 accepted to enter the study and were randomized. Among them, 109 accepted to carry out additional analysis included in the sub-study.

- 89 subjects dropped out while 236 volunteers (82 in the sub-study) completed the 12-week trial, and subjects with at least 70% compliance to consumption for each administered food were included in the per protocol (PP) statistical analysis.
- In volunteers with low HDL-c (≤50 mg/dl for females, ≤40 mg/dl for males) at baseline, all BEF significantly increase HDL-c (the so-called "good cholesterol"). The positive effect of BEF was already achieved after 6 weeks of consumption, and it lasted over the whole period of treatment.
- The HDL-c increasing effect was more evident using BEF enriched with DHA+AC.
- The group receiving DHA+OBG experienced a clear reduction of LDL-c level (the so-called "bad cholesterol").
- Consumption of all food enriched with DHA was associated to significant reduction of systolic blood pressure. The effect appeared mainly ascribable to administration of DHA+OBG and DHA+AC embedded in pancakes.
- A general guideline for conducting nutritional trials was made public.

Highlights

- The rs17300539 SNP on the ADIPOQ gene was found associated with the risk of MetS in all national sub-cohorts.
- NMR-based metabolomics analyses could discriminate samples collected at the beginning and the end of the study with high accuracy, confirming that treatment has an impact on the metabolome.
- We detected significant changes in the lipoprotein profiles in serum of volunteers receiving DHA+AC embedded in the bakery product, but not in the egg product.
- Genes associated with epigenetic alterations induced by the BEF were enriched in binding sites for a series of transcription factors with reported functions in adipose tissue function.
- An increase in some microbial groups induced by BEF consumption seemed to exclude the increase of pathogenic members of the same group of bacteria, suggesting an ecological barrier function.

Highlights

- Since *in vivo* foods are digested and some bioactives are extensively metabolized so that the effective molecules are very different from parent compounds, cells were supplemented with the main metabolites of bioactive used to formulate BEF.
- Cells were supplemented using bioactive concentrations within the physiological range, and possible cytotoxicity was evaluated prior to other experiments.
- In cultured hepatocytes, a "DHA signature" was clearly evidenced, indicating that DHA has the greatest impact on the transcriptome, lipidome and metabolome. Cholesterol and fatty acid metabolism were the main pathways regulated by DHA. PRO and PCA modulated DHA effect, while their impact when supplemented alone was small.
- In hepatocytes, the epigenetic effect of DHA was related more to chromatin changes than DNA methylation and was consistent with the observed modulation of cholesterol and fatty acid metabolism.
- A new strategy for administration of DHA and other fatty acids to human adipocytes was developed.
- DHA appeared by far the bioactive with the greatest impact on the adipocyte transcriptome, promoting adipocyte function. Again, PRO and PCA displayed positive or negative cross-talk with DHA.
- In adipocytes, DHA and combinations induced strong changes in DNA methylation, while PCA and PRO had little effects on this epigenetic mark.

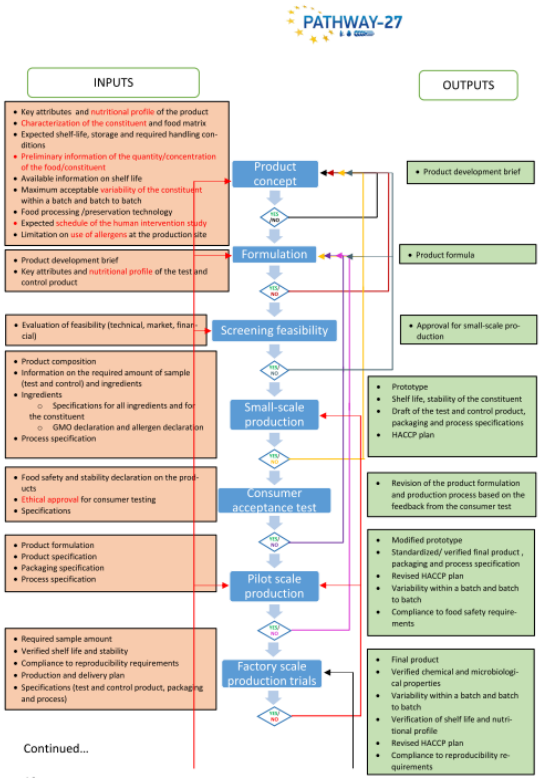

Figure 5. Aspects addressed by the Guidelines for the food industry

Figure 6. The PATHWAY-27 Guidance Paper Workshps

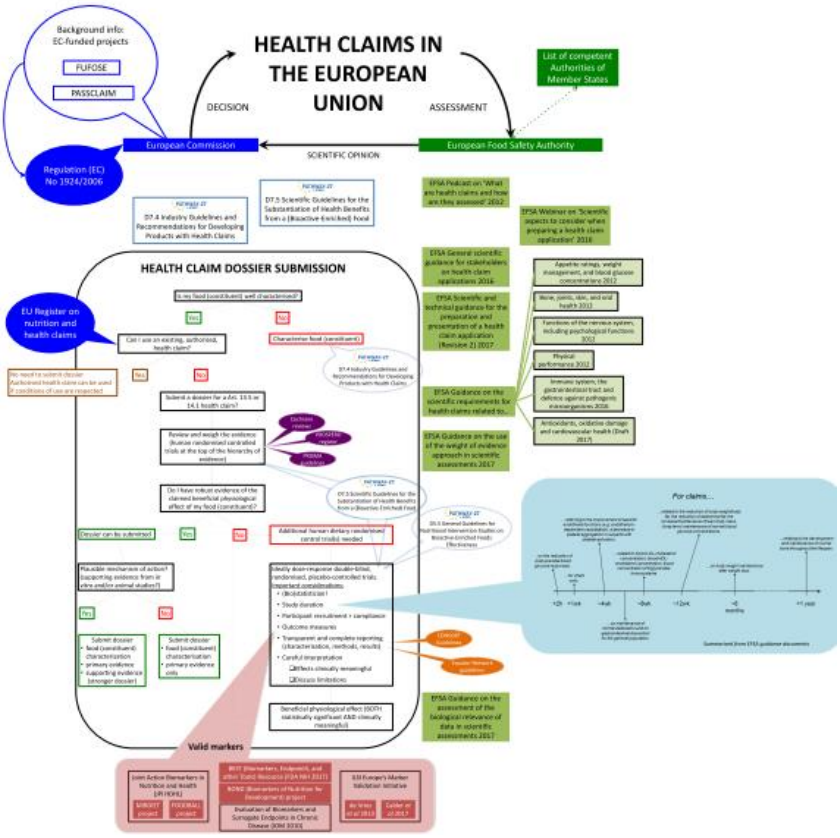

Figure 7. The interactive diagram of the regulatory framework

Highlights

- A set of guidelines (**Guidelines for the scientific community**) addressed to scientists from both academia and the food industry was developed.
- Another set of guidelines (**Guidelines for the food industry)** offering a structured product development approach addressing all aspects that SMEs and their suppliers should consider when designing products with health claims in Europe was also developed.
- The starting step of the Guidelines for the food industry was to conduct a survey amongst relevant stakeholders to collect and identify industry/SME needs and difficulties in establishing and submitting health-claims.
- Finalisation of both Scientific and Industry Guidelines was based on interaction with key stakeholders. The Guidance Paper Workshops held in Brussels in September 2017 allowed reviewing the Guidelines before publication.
- An additional set of information supporting the implementation of the European legislation frameworks on health claims was also prepared.

Figure 8. The PATHWAY-27 project logo

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