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Lactobacillus plantarum 299v and an increase of non-haem iron absorption: evaluation of a health claim pursuant to Article 13(5) of Regulation (EC) No 1924/2006

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)

Abstract

Following an application from Probi AB submitted for authorisation of a health claim pursuant to Article 13(5) of Regulation (EC) No 1924/2006 via the Competent Authority of Sweden, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on the scientific substantiation of a health claim related to *Lactobacillus plantarum* 299v and an increase of non-haem iron absorption. The food that is the subject of the health claim is '*Lactobacillus plantarum* 299v (Lp299v)'. The Panel considers that Lp299v is sufficiently characterised. The claimed effect proposed by the applicant is 'increase of non-haem iron absorption'. The target population proposed by the applicant is 'healthy adults who want to increase their iron uptake'. The Panel considers that increasing non-haem iron absorption is a beneficial physiological effect. In weighing the evidence, the Panel took into account that the results of two double-blind, placebo-controlled, cross-over studies are inconsistent, as one study with some methodological limitations showed a positive effect of Lp299v on non-haem absorption, whereas the other did not show an effect. The Panel noted that among four single-blind, placebo-controlled, sequential studies at risk of systematic bias, three studies showed a positive effect of Lp299v on non-haem absorption and one did not show an effect. The Panel also took into account that there is no evidence for a plausible mechanism by which Lp299v could increase non-haem iron absorption *in vivo* in humans. The Panel concludes that the scientific evidence is insufficient to establish a cause and effect relationship between the consumption of Lp299v and an increase of non-haem iron absorption.

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Keywords: *Lactobacillus plantarum* 299v, non-haem iron, absorption, health claim

Requestor: Competent Authority of Sweden following an application by Probi AB

Question number: EFSA-Q-2015-00696

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Panel members: Jean-Louis Bresson, Barbara Burlingame, Tara Dean, Susan Fairweather-Tait, Marina Heinonen, Karen Ildico Hirsch-Ernst, Inge Mangelsdorf, Harry McArdle, Androniki Naska, Monika Neuhäuser-Berthold, Grażyna Nowicka, Kristina Pentieva, Yolanda Sanz, Alfonso Siani, Anders Sjödin, Martin Stern, Daniel Tomé, Dominique Turck, Hendrik Van Loveren, Marco Vinceti and Peter Willatts

Competing interests: In line with EFSA's policy on declarations of interest, Panel member Anders Sjödin did not participate in the development and adoption of this scientific opinion.

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Summary

Following an application from Probi AB submitted for authorisation of a health claim pursuant to Article 13(5) of Regulation (EC) No 1924/2006 via the Competent Authority of Sweden, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on the scientific substantiation of a health claim related to *Lactobacillus plantarum* 299v and an increase of non-haem iron absorption.

The scope of the application was proposed to fall under a health claim based on newly developed scientific evidence. The application included a request for the protection of proprietary data.

The general approach of the NDA Panel for the evaluation of health claims applications is outlined in the European Food Safety Authority (EFSA) general guidance for stakeholders on the evaluation of Article 13.5 and 14 health claims (EFSA NDA Panel, 2016a) and the guidance on the scientific requirements for health claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms (EFSA NDA Panel, 2016b).

The food that is the subject of the health claim is '*Lactobacillus plantarum* 299v (Lp299v)'. The Panel considers that Lp299v is sufficiently characterised.

The claimed effect proposed by the applicant is 'increase of non-haem iron absorption'. The target population proposed by the applicant is 'healthy adults who want to increase their iron uptake'. The Panel considers that an increase of non-haem iron absorption is a beneficial physiological effect.

A total of six human intervention studies (in five reports/publications) which had investigated the effect of Lp299v on non-haem iron absorption in adults were submitted by the applicant. Two human intervention studies had a randomised, double-blind, cross-over design. The Panel notes that, out these studies, one with some methodological limitations showed a positive effect of Lp299v on non-haem iron absorption and one did not show an effect. The Panel considers that results of these studies are inconsistent. The remaining four human intervention studies submitted by the applicant had a single-blind, sequential design in which each subject served as its own control and all subjects received the control food first. The Panel notes that, among these studies, three showed a positive effect of Lp299v on non-haem iron absorption and one did not show an effect. The Panel considers, however, that the sequential, non-randomised order of administration of the control and test foods may have introduced a systematic bias to the results of these studies.

The applicant also provided three *in vitro* studies in relation to the mechanism by which Lp299v could exert the claimed effect. Based on the information provided, the Panel considers that there is no evidence for a plausible mechanism by which Lp299v could increase non-haem iron absorption.

In weighing the evidence, the Panel took into account that the results of two double-blind, placebo-controlled, cross-over studies are inconsistent, as one study with some methodological limitations showed a positive effect of Lp299v on non-haem absorption, whereas the other did not show an effect. The Panel noted that among four single-blind, placebo-controlled, sequential studies at risk of systematic bias, three studies showed a positive effect of Lp299v on non-haem absorption and one did not show an effect. The Panel also took into account that there is no evidence for a plausible mechanism by which Lp299v could increase non-haem iron absorption *in vivo* in humans.

On the basis of the data presented, the Panel concludes that the scientific evidence is insufficient to establish a cause and effect relationship between the consumption of Lp299v and an increase of non-haem iron absorption.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1924/2006[1] harmonises the provisions that relate to nutrition and health claims, and establishes rules governing the Community authorisation of health claims made on foods. As a rule, health claims are prohibited unless they comply with the general and specific requirements of this Regulation, are authorised in accordance with this Regulation, and are included in the lists of authorised claims provided for in Articles 13 and 14 thereof. In particular, Article 13(5) of this Regulation lays down provisions for the addition of claims (other than those referring to the reduction of disease risk and to children's development and health), which are based on newly developed scientific evidence, or which include a request for the protection of proprietary data, to the Community list of permitted claims referred to in Article 13(3).

According to Article 18 of this Regulation, an application for inclusion in the Community list of permitted claims referred to in Article 13(3) shall be submitted by the applicant to the national competent authority of a Member State, which will make the application and any supplementary information supplied by the applicant available to the European Food Safety Authority (EFSA).

1.2. Interpretation of the Terms of Reference

EFSA is requested to evaluate the scientific data submitted by the applicant in accordance with Article 16(3) of Regulation (EC) No 1924/2006. On the basis of that evaluation, EFSA will issue an opinion on the scientific substantiation of a health claim related to: *Lactobacillus plantarum* 299v (Lp299v) and an increase of non-haem iron absorption.

The present opinion does not constitute, and cannot be construed as, an authorisation for the marketing of Lp299v, a positive assessment of its safety nor a decision on whether Lp299v is, or is not, classified as a foodstuff. It should be noted that such an assessment is not foreseen in the framework of Regulation (EC) No 1924/2006.

It should also be highlighted that the scope, the proposed wording of the claim, and the conditions of use as proposed by the applicant may be subject to changes, pending the outcome of the authorisation procedure foreseen in Article 18(4) of Regulation (EC) No 1924/2006.

1.3. Additional information

A claim on Lp299v and improve iron absorption has already been assessed by the Panel with unfavourable outcome (EFSA NDA Panel, 2009).

2. Data and methodologies

2.1. Data

2.1.1. Information provided by the applicant

2.1.1.1. Food constituent as stated by the applicant

According to the applicant, the food that is the subject of the claim is Lp299v (DSM 9843), which was isolated from human intestinal mucosa and is included in a genetic subgroup of the species *L. plantarum*.

2.1.1.2. Health relationship as claimed by the applicant

According to the applicant, the claimed effect relates to the increase of non-haem iron absorption.

2.1.1.3. Wording of the health claim as proposed by the applicant

The applicant has proposed the following wording for the health claim: '*Lactobacillus plantarum* 299v increases non-haem iron absorption'.

[1] Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. OJ L 404, 30.12.2006, p. 9–25.

2.1.1.4. Specific conditions of use as proposed by the applicant

According to the applicant, the claimed effect is based on a daily intake of 10^9–10^{11} colony forming unit (cfu) Lp299v. This amount can be consumed as part of a meal-included in a food product (e.g. a fruit drink) or taken as a food supplement (e.g. a capsule with freeze-dried bacteria). The proposed target population is 'healthy adults who want to increase their iron uptake'.

2.1.1.5. Data provided by the applicant

The applicant provided a health claim application on Lp299v and increase of non-haem iron absorption pursuant to Article 13.5 of Regulation 1924/2006. The application was presented in a common and structured format as outlined in the Scientific and technical guidance for the preparation and presentation of applications for authorisation of health claims (EFSA NDA Panel, 2011).

As outlined in the EFSA general guidance for stakeholders on health claim applications (EFSA NDA Panel, 2016a), it is the responsibility of the applicant to provide the totality of the available evidence.

2.2. Methodologies

The general approach of the NDA Panel for the evaluation of health claim applications is outlined in the EFSA general guidance for stakeholders on health claim applications (EFSA NDA Panel, 2016a).

The scientific requirements for health claims related to functions of the gastrointestinal tract are outlined in a specific EFSA guidance (EFSA NDA Panel, 2016b).

3. Assessment

3.1. Characterisation of the food constituent

The food which is the subject of the health claim is *Lactobacillus plantarum* 299v (Lp299v).

Lp299v has been identified at species and strain level by both phenotypic and genotypic methods (Johansson et al., 1993, 1995). Data on the specifications as well as information on stability of the strain in freeze-dried powder or frozen concentrates are provided in the application. A culture collection number from the German culture collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen - DSM 9843) is indicated for the strain. Information about the manufacturing process, stability and survival in the digestive tract was provided in the application.

Lp299v can be measured in foods by established methods.

The Panel considers that Lp299v is sufficiently characterised.

3.2. Relevance of the claimed effect to human health

The claimed effect proposed by the applicant is 'increase of non-haem iron absorption'. The target population proposed by the applicant is 'healthy adults who want to increase their iron uptake'.

Iron deficiency is one of the most common micronutrient deficiencies in the European Union (EU), and can result in anaemia. Non-haem iron is generally not well absorbed in the human intestine, and can be a limiting factor for the maintenance of adequate iron status (EFSA NDA Panel, 2016a).

The Panel considers that an increase of non-haem iron absorption is a beneficial physiological effect.

3.3. Scientific substantiation of the claimed effect

The applicant performed a literature search in PubMed, the Cochrane Library, and Web of Science with the following key words: *Lactobacillus plantarum* 299v, *Lactobacillus plantarum*, *Lactobacillus*, *plantarum*, lactic acid bacteria, probiotics, Lp299v, DMS 9843, iron absorption. No limits were applied.

A claim submitted under the Article 13(5) procedure on Lp299v (DSM 9843) and increasing non-haem iron absorption was evaluated by the Panel with an unfavourable opinion on the basis of the weaknesses of the four human intervention studies which were submitted for substantiation (EFSA NDA Panel, 2009).

3.3.1. Human intervention (efficacy) studies

A total of six human intervention studies (in five reports/publications) which had investigated the effect of Lp299v on non-haem iron absorption in adults have been submitted by the applicant in the present application. These include the four human intervention studies (in three reports/publications) evaluated previously by the Panel (Hoppe et al., 2015 – submitted in the previous application as

Hulthen and Hoppe 2007, unpublished; Bering et al., 2006, 2007) and two human studies (Hulthen and Hoppe, 2014a,b - unpublished) not included in the previous application (EFSA NDA Panel, 2009).

In the aforementioned studies, iron absorption was measured using the dual-label extrinsic tag technique, in which test meals are extrinsically labelled with radioisotopes ^{55}Fe or ^{59}Fe, and iron absorption is calculated from the radioisotope activity in whole blood (red blood cell haemoglobin) approximately 10–18 days after the test meal. All labelled meals were consumed after an overnight fast.

Two human intervention studies had a randomised, double-blind, cross-over design (Bering et al., 2006, 2007).

Bering et al. (2006) recruited 24 women (25 ± 4 years) with serum ferritin concentrations ranging from 12 to 40 μg/L. Four different products were assessed (one test and three control products). The test product (A) consisted of 100 g oat gruel fermented with Lp299v (1.1×10^9 cfu/g). The control products were (B) 100 g pasteurised fermented oat gruel, (C) a non-fermented gruel (pH adjusted with lactic acid) and (D) a non-fermented gruel with added lactic and acetic acids. Two products were administered in each period. In the first period, the first two foods (2 days for each food) were tested for four consecutive days (foods A and B). The participants were randomised to one of the six possible sequences (i.e. ABBA, ABAB, AABB, BAAB, BABA and BBAA). After 18 days, the other two foods (C and D) were tested in the same way, by randomising participants to one of the six possible sequences (i.e. CDDC, CDCD, CCDD, DCCD, DCDC and DDCC). Iron absorption was measured using ^{59}Fe to label the native iron in the gruel. Iron absorption from the distal part of the intestine was assessed by giving ^{55}Fe in enterocoated capsules designed to disintegrate in the ileum with the gruel meal. The retention of ^{59}Fe was measured in a whole-body counter at baseline and 15 days after intake of the test meals, and the activity of both isotopes, ^{55}Fe and ^{59}Fe, was measured in a blood sample drawn 18 days after intake of the test meals. Residual isotope activities from the first period were subtracted from the isotope activity levels in the second period and iron excretion between the two measurements was assumed to be negligible. Iron absorption data were expressed as ^{59}Fe whole-body retention measured directly from whole-body counting, as ^{55}Fe whole-body retention determined from the relative activities of ^{55}Fe and ^{59}Fe in blood, and the ^{59}Fe whole-body retention, assuming that the fractions of the two isotopes in blood are similar.

Non-haem iron absorption was calculated using a linear mixed model with log non-haem iron absorption as dependent variable, meal and ferritin as independent fixed variables, and subject and subject x period interaction as random effects. Non-haem iron absorption was significantly higher from the gruel containing Lp299v (A) (1.1%, 95% CI 0.8, 1.5) than from the pasteurised fermented gruel (B) (0.6%, 95% CI 0.4, 0.7), the pH-adjusted non-fermented gruel (C) (0.5%, 95% CI 0.4, 0.7) and (D) the non-fermented gruel with organic acids (0.5%, 95% CI 0.4, 0.7) ($p < 0.0001$). There was no detectable iron absorption from the distal small intestine.

The Panel notes that there were differences in the composition of the test (A) and control (B, C and D) products. The pasteurised fermented oat gruel (B) contained 19% and 8% lower concentrations of lactic and acetic acids, respectively, than the test product (A), and non-fermented controls contained 45% and 72% (C) and 60% and 7% (D) lower lactic and acetic content, respectively, than the test product (A). The authors suggest that differences in the content of organic acids alone between the test and control foods cannot explain the observed differences in iron absorption. However, the Panel notes that the relative effects of Lp299v vs differences in organic acids on non-haem iron absorption have not been quantified. The Panel considers that this study with some methodological limitations (e.g. test and control foods differ in characteristics other than the Lp299v content which may have affected non-haem iron absorption) shows an effect of Lp299v, when consumed in a single meal of fermented oat gruel at a dose of 10^{11} cfu/g daily for 2 days, on non-haem iron absorption.

Bering et al. (2007) undertook a second randomised, double-blind, cross-over study to investigate the effect of Lp299v on iron absorption from fermented oat gruel. In this study, 18 women (22 ± 3 years) with serum ferritin concentrations ranging from 13 to 29 μg/L were recruited. Sample size was calculated using data from the previous study to detect a 2% change in iron absorption at a significance level of 0.01 with a power of 90%. Volunteers consumed, on two consecutive days, either 100 g of a heat-inactivated lactic acid-fermented oat gruel with added lyophilised viable Lp299v (10^9 cfu/g added 1 day before use) or the same product without Lp299v. Both foods were consumed together with a whole-wheat roll and butter. After 18 days, the volunteers received the other product. The total iron content of the test meal was 1.9 mg. Non-haem Fe absorption from the two test meals was determined by extrinsic labelling of the oat gruels with ^{59}Fe, while absorption from the distal part of the intestine was assessed by ^{55}Fe in enterocoated capsules designed to disintegrate in the ileum.

Mean non-haem iron absorption was 1.4% (95% CI 0.9, 2.2) and 1.3% (95% CI 0.9, 2.0) from the gruels with and without added lyophilised viable Lp299v, respectively. No detectable absorption of iron was observed in the ileum or colon. The Panel considers that this study does not show an effect of Lp299v at a dose of 10^9 cfu/g daily for 2 days on non-haem iron absorption.

The authors proposed an explanation for the different results obtained in the studies by Bering et al. (2006,2007), which is that the bacteria used in the test meals were not in a comparable active state. In the first study, the concentration of lactic acid in the fermented gruel with Lp299v increased by 19% when it was stored at 4°C for 25 days between the two test periods, and the Lp299v was metabolically active during the fermentation process. In the second study, Lp299v was added and lyophilised to a cold product in order to avoid changes in the composition of the test gruel. A *post hoc* examination of the gruel showed that the metabolic activity of Lp299v was retarded by approximately 1 h when added in a lyophilised form, which could potentially affect the metabolic activity in the duodenum, the primary site of iron absorption.

The Panel notes that, out of the two double-blind, placebo-controlled, cross-over, human intervention studies provided, one with some methodological limitations showed a positive effect of Lp299v on non-haem iron absorption (Bering et al., 2006) and one did not show an effect (Bering et al., 2007). The Panel considers that results of these studies are inconsistent.

The remaining four human intervention studies submitted by the applicant (in three reports/ publications) had a single-blind, sequential design in which each subject served as its own control and all subjects received the control food first (Hulthen and Hoppe, 2014a,b and Hoppe et al., 2015).

Hulthen and Hoppe (2014a, unpublished, claimed as proprietary by the applicant) recruited 18 women aged 26.2 ± 4.6 years with a mean serum ferritin concentration of 30 ± 21 μg/L (range 8–80 μg/L). On two consecutive days, each subject took three capsules. One capsule contained 4.2 mg iron (ferrous fumarate), 30 μg folic acid and 12 mg ascorbic acid. The other two capsules contained ^{55}Fe (to label the ferrous fumarate) and potato starch. The three capsules were taken half-way through a meal (two wheat rolls with margarine and marmalade) in order to maximise the mixing process and exchange of isotopes with iron in the meal. On the following 2 days, each woman consumed similar capsules but with 10^{10} cfu freeze-dried Lp299v added to the first capsule, and ^{59}Fe (to label the ferrous fumarate) in the other two capsules. The three capsules were taken again half-way through the standardised meal. The control and test capsules (with/without Lp299v) were given twice (on two consecutive days) in order to minimise day-to-day variations in iron absorption. Approximately 14 (range 10–16) days later, the concentration of ^{55}Fe and ^{59}Fe in whole blood was measured by liquid scintillation counting and iron absorption was determined as the % of the dose administered that was present in whole blood, calculated from the individual's height, weight and haemoglobin concentration. In order to adjust for interindividual differences in efficiency of absorption, the % absorption from a 3 mg reference dose of ^{59}Fe-labelled ferrous ascorbate (given after an overnight fast on two consecutive days) was measured and the values obtained from the test meals were normalised to 40% iron absorption from the reference dose.

Results were provided for 14 out of the 18 women recruited (one woman withdrew at the blood sampling stage and three were excluded from data analysis due to very high serum ferritin concentrations (n = 1), on-going infection (n = 1) and involvement in a previous isotope study that might have affected the results (n = 1)). Mean non-haem iron absorption values, adjusted to the 40% absorption of a reference dose, were 22.4% (SD 17.3%) from the meal with Lp299v and 17.4% (SD 13.4%) from the control meal, respectively (p = 0.04). The Panel notes that iron absorption from 4.2 mg iron as ferrous fumarate was increased in women by 29% (from 17.4% to 22.4%) when given with Lp299v at a dose of 10^{10} cfu. The Panel considers that this study shows an effect of Lp299v, when consumed in capsules at a dose 10^{10} cfu with a single meal on two consecutive days, on non-haem iron absorption in women with mean serum ferritin concentration ranging from 8 to 80 μg/L. The Panel notes, however, that the non-randomised design of the study might have introduced a systematic bias in the results.

Hulthen and Hoppe (2014b, unpublished, claimed as proprietary by the applicant) recruited 36 women aged 26.2 ± 4.6 years with a mean serum ferritin concentration of 27 ± 14 μg/L. Serum ferritin concentration < 60 μg/L was an inclusion criteria. The study followed exactly the same protocol as the previous study. Data were presented for 28 out of the 36 women recruited (two women withdrew and six were excluded because of a serum ferritin concentration > 60 μg/L (n = 3) or CRP concentrations ≥ 5 mg/L (n = 3)).

Mean non-haem iron absorption values, adjusted to the 40% absorption of a reference dose, were 24.5% (SD 12.0%) from the meal with Lp299v and 20.9% (SD 13.1%) from the control meal,

respectively (p = 0.003). The Panel notes that iron absorption from 4.2 mg iron as ferrous fumarate was increased by 17% (from 20.9% to 24.5%) in women with a mean serum ferritin concentration of 27 μg/L (range 10–54 μg/L) when given with Lp299v at a dose of 10^{10} cfu. The Panel considers that this study shows an effect of Lp299v, when consumed in capsules at a dose 10^{10} cfu for two consecutive days, on non-haem iron absorption. The Panel notes, however, that the non-randomised design of the study might have introduced a systematic bias in the results.

Hoppe et al. (2015) tested the effect of adding Lp299v to an oat-based fruit drink (containing grape, mango, passion fruit, banana and added sugar) which was supplemented with iron as ferrous lactate (2.1 mg/100 mL) and ascorbic acid (50 mg/100 mL). Two single-blind, sequential, non-randomised studies were performed, one giving a dose of 10^9 cfu Lp299v (study 1) and the other a dose of 10^{10} cfu Lp299v (study 2). The protocols were similar to that used by Hulthen and Hoppe (2014a), with the iron in the fruit drinks given with Lp299v being extrinsically labelled with ^{59}Fe and the iron in the fruit drinks given without Lp299v being labelled with ^{55}Fe, but the radioisotope measurements were different. Iron absorption from the ^{59}Fe-labelled fruit drink was measured using a whole-body counter and iron absorption was expressed as % of the dose administered after correcting for physical decay and background radioactivity. After whole-body counting, a blood sample was drawn and the relative absorption of each of the two isotopes was determined using liquid scintillation counting. This relative absorption was used, together with the blood volume calculated from each individual's height, weight and haemoglobin concentration, to calculate ^{55}Fe absorption. In order to adjust for interindividual differences in efficiency of absorption, the % absorption from a 3 mg reference dose of ^{59}Fe-labelled ferrous ascorbate (given after an overnight fast on two consecutive days) was measured using whole-body counting.

In the first study, 10 women (22–40 years) with mean serum ferritin concentration of 33 ± 13 μg/L consumed 200 mL of the fruit drink (supplemented with 2.1 mg/100 mL of iron as ferrous lactate and 50 mg/100 mL of ascorbic acid) for two consecutive days and then the same fruit drink with Lp299v (10^9 cfu/200 mL) on the subsequent two consecutive days. Iron absorption, normalised to the 40% absorption of the reference dose, from the drink containing Lp299v was 28.6% (SD 12.5%) and from the control drink was 18.5% (SD 5.8%) (p = 0.0284 – calculated from individual data provided by the applicant).

The second study followed the same design (11 women, 22–40 years with mean serum ferritin concentration of 33 ± 14 μg/L) except that the test product was supplemented with a higher dose of Lp299v (10^{10} cfu/200 mL). Iron absorption from the drink containing Lp299v was 29.1% (SD 17.0%) and from the control drink was 20.1% (SD 6.4%) (p = 0.08 – calculated from individual data provided by the applicant).

Iron absorption from the two drinks containing Lp299v and from the two control drinks in the two studies was not significantly different (p = 0.941 and p = 0.558, respectively – calculated from individual data provided by the applicant). The Panel notes that pooling of results from the two studies was not preplanned, and that the two studies are not identical because the composition of the products is not exactly the same and the dose of Lp299v is higher in the second trial. The Panel considers, therefore, that the results of these two studies should be evaluated separately. The Panel considers that one study (study 1) shows an effect of Lp299v on non-haem iron absorption when consumed as an oat-based fruit drink at a dose 10^9 cfu/200 mL daily in a single meal for two consecutive days, whereas the effect was not observed at doses of 10^{10} cfu/200 mL (study 2). The Panel notes that the non-randomised design of the study might have introduced a systematic bias in the results.

The Panel notes that the three human intervention studies described above (and reported in three reports/publications: Hulthen and Hoppe, 2014a,b; Hoppe et al., 2015) were designed as single-blind, non-randomised, sequential studies without washout periods. The control foods (without Lp299v) were given on the first two consecutive days, followed by the test foods (with Lp299v) on days 3 and 4. Upon a request for clarification from EFSA, the applicant explained that the fixed order of administration of the test and control meals (control first followed by the test meal containing Lp299v) was applied owing to a potential carry-over effect, which could be expected from the adherence of Lp299v to the intestinal epithelium (Johansson et al., 1993; Bering et al., 2006).

The Panel notes the explanation and rationale provided by the applicant for choosing non-randomised sequential designs. The Panel considers, however, that the sequential, non-randomised order of administration of the control and test foods might introduce a systematic bias to the results of these studies.

Ingestion of ^{55}Fe in these studies was always associated with the control meals, while ^{59}Fe was always associated with the food containing Lp299v. Taking into account that the recovery of these isotopes may be different and that the order of ingestion of the isotopes was not randomised, this may constitute a source of systematic bias. Upon a request for clarification from EFSA, the applicant explained that some modifications of the classical Eakins and Brown (1966) methods were applied in these studies (e.g. increased amount of iron in the samples to reduce the variability, reduced number of cleaning steps to improve the recovery, and replacement of the scintillation fluid from Cab-o-Suil to Ultima Gold AB to reduce cross-counting of ^{59}Fe into the ^{55}Fe window). The applicant claimed that the serving order of the isotopes did not have an effect on the final result (Brise and Hallberg, 1962; unpublished study by Lena Hulthen, personal communication). Based on the information provided by the applicant, the Panel considers that the labelling of the test and control meals with different Fe isotopes (^{59}Fe and ^{55}Fe, respectively) in these studies is unlikely to have introduced additional systematic bias in the results.

The Panel notes that, out of the four single-blind, placebo-controlled, sequential human intervention studies provided, three studies showed a positive effect of Lp299v on non-haem iron absorption (Hulthen and Hoppe, 2014a,b; Hoppe et al., 2015 at the dose 10^9 cfu) and one did not show an effect (Hoppe et al., 2015 at the dose 10^{10} cfu). The Panel considers, however, that the sequential, non-randomised order of administration of the control and test foods may have introduced a systematic bias to the results of these studies.

3.3.1.1. Summary of human intervention (efficacy) studies

The Panel notes the differences in the form of Lp299v used in the human studies submitted (freeze-dried, liquid), in the food matrix, and in the mode of administration. For example, Lp299v was added to a heat-treated fermented oat gruel of low iron bioavailability (Bering et al., 2007), to a fruit drink of high-iron bioavailability (Hoppe et al., 2015), and was given in a capsule (which also contained ascorbic acid, folic acid and iron) consumed with two wheat rolls (Hulthen and Hoppe, 2014a,b). Some studies measured native iron absorption (Bering et al., 2006, 2007), whereas others measured absorption from a mixture of native plus added iron (Hulthen and Hoppe 2014a,b, Hoppe et al., 2015). The dose of Lp299v was 10^9 cfu (Hoppe et al., 2015), 10^{10} cfu (Hulthen and Hoppe, 2014a,b; Hoppe et al., 2015) and 10^{11} cfu (Bering et al., 2006, 2007). The dose of iron ranged from 1.9 to 5.4 mg/test meal.

In these studies, there was a wide range in % iron absorption. In the oat gruels containing 2.8 mg native iron, absorption was 1.1% with Lp299v and 0.6% without Lp299v (Bering et al., 2006), which equates to an absorption of 0.031 and 0.017 mg of iron, respectively. In the fruit drink containing approximately 5 mg iron as ferrous lactate, iron absorption was 29.1% with added Lp299v and 20.1% without Lp299v (Hoppe et al., 2015), which equates to an absorption of 1.51 and 1.09 mg of iron, respectively.

The Panel considers that the results of two double-blind, placebo-controlled, cross-over studies are inconsistent, as one with some methodological limitations showed a positive effect of Lp299v on non-haem iron absorption (Bering et al., 2006) and one did not show an effect (Bering et al., 2006). The Panel notes that among four single-blind, placebo-controlled, sequential studies at risk of systematic bias, three studies showed a positive effect of Lp299v on non-haem iron absorption (Hulthen and Hoppe, 2014a,b; Hoppe et al., 2015 at the dose 10^9 cfu) and one did not show an effect (Hoppe et al., 2015 at the dose 10^{10} cfu).

3.3.2. Mechanism by which the food constituent could exert the claimed effect

Three *in vitro* studies were provided by the applicant in support of the mechanism by which the food could exert an effect (Sandberg, 2006; Scheers and Sandberg, 2015a,b).

An *in vitro* study by Sandberg (2006, unpublished, claimed as proprietary by the applicant) assessed iron (^{55}Fe) uptake and transepithelial transport in Caco-2 cells (colonic cells) treated with *L. plantarum* strains. The Panel notes that there is no information about the control used and the quantity of iron to which the cells were exposed. The Panel also notes that standard deviations for the % transport data are not provided. The Panel assumes that this study was designed to measure the movement of added ^{55}Fe in the presence (or absence) of different strains of bacteria, showing that the Lp299v mutant that lacks the capacity to bind mannose behaved similarly to the other strains with respect to ^{55}Fe transport.

Scheers and Sandberg (2015a, unpublished, claimed as proprietary by the applicant) examined the effect of Lp299v on iron oxidation state in meals going through an *in vitro* simulated digestion. The

Panel notes that the results of this study do not provide an explanation for the enhancing effect of Lp299v on non-haem iron absorption.

Scheers and Sandberg (2015b, unpublished, claimed as proprietary by the applicant) measured the uptake of iron (from measurements of ferritin concentration) in Caco-2 cells cocultured with mucus-producing goblet cells and reported no difference in ferritin concentration in cells exposed to the capsule containing Lp299v compared with the control (no Lp299v) over 60 min.

The Panel considers that these studies do not provide any evidence for mechanism(s) by which Lp299v could increase non-haem iron absorption *in vivo* in humans.

3.3.3. Weighing of the evidence

In weighing the evidence, the Panel took into account that the results of two double-blind, placebo-controlled, cross-over studies are inconsistent, as one study with some methodological limitations (Bering et al., 2006) showed a positive effect of Lp299v on non-haem iron absorption, whereas the other (Bering et al., 2007) did not show an effect. The Panel noted that among four single-blind, placebo-controlled, sequential studies at risk of systematic bias, three studies showed a positive effect of Lp299v on non-haem iron absorption (Hulthen and Hoppe, 2014a,b; Hoppe et al., 2015 at the dose 10^9 cfu) and one did not show any effect (Hoppe et al., 2015 at the dose 10^{10} cfu). The Panel also took into account that there is no evidence for a plausible mechanism by which Lp299v could increase non-haem iron absorption *in vivo* in humans.

The Panel concludes that the evidence provided is insufficient to establish a cause and effect relationship between the consumption of Lp299v and an increase of non-haem iron absorption.

4. Conclusions

On the basis of the data presented, the Panel concludes that:

- The food, Lp299v, which is the subject of the health claim, is sufficiently characterised.
- The claimed effect proposed by the applicant is 'increase of non-haem iron absorption'. The target population proposed by the applicant is 'healthy adults who want to increase their iron uptake'. Improving non-haem iron absorption is a beneficial physiological effect.
- Scientific evidence is insufficient to establish a cause and effect relationship between the consumption of Lp299v and an increase of non-haem iron absorption.

Steps taken by EFSA

1) Health claim application on *Lactobacillus plantarum* 299v and an increase of non-haem iron absorption pursuant to Article 13(5) of Regulation (EC) No 1924/2006 (Claim serial No: 0441_SE). Submitted by Probi AB, Ideon Gamma 1, S22370 Lund, Sweden.
2) This application was received by EFSA on 9/11/2015.
3) The scope of the application was proposed to fall under a health claim based on newly developed scientific evidence. The application included a request for the protection of proprietary data.
4) The scientific evaluation procedure started on 7/1/2016.
5) On 19/1/2016, the Working Group on Claims of the NDA Panel agreed on a list of questions for the applicant to provide additional information to accompany the application. The scientific evaluation was suspended on 28/1/2016 and was restarted on 10/2/2016, in compliance with Article 18(3) of Regulation (EC) No 1924/2006.
6) On 11/2/2016, EFSA received the applicant's reply.
7) On 20/4/2016, the Working Group on Claims of the NDA Panel agreed on a list of questions for the applicant to provide additional information to accompany the application. The scientific evaluation was suspended on 27/4/2016 and was restarted on 11/5/2016, in compliance with Article 18(3) of Regulation (EC) No 1924/2006.
8) On 11/5/2016, EFSA received the applicant's reply.
9) During its meeting on 28/6/2016, the NDA Panel, having evaluated the data, adopted an opinion on the scientific substantiation of a health claim related to *Lactobacillus plantarum* 299v and an increase of non-haem iron absorption.

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Abbreviations

cfu	colony forming units
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lp	*Lactobacillus plantarum*
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies