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Health risk assessment of *Bifidobacterium breve* M-16V in infant formula

Gry Irene Granli Schultz, Christine Louise Parr, Pål Trosvik, Siamak Yazdankhah, Lene Frost Andersen, Knut Tomas Dalen, Lars Thore Fadnes, Ingrid Kvestad, Lise Madsen, Anine Christine Medin, Kjetil K. Melby, Vibeke H. Telle-Hansen, Inger Aakre, Tor A. Strand

Scientific Opinion of the Panel on Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food and Environment

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Preparation of the opinion

The Norwegian Scientific Committee for Food and Environment (Vitenskapskomiteen for mat og miljø, VKM) appointed a project group to draft the opinion. The project group consisted of three VKM members and two VKM staff. The Committee, by an interdisciplinary VKM approval group appointed specifically for the assignment, assessed, and approved the final opinion.

Authors of the opinion

The authors have contributed to the opinion in a way that fulfils the authorship principles of VKM (VKM, 2023). The principles reflect the collaborative nature of the work, and the authors have contributed as members of the project group and/or an interdisciplinary VKM approval group, appointed specifically for the assignment.

Members of the project group

(in alphabetical order after chair of the project group)

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Acknowledgement

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third-party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

Background: VKM, commissioned by the Norwegian Food Safety Authority (NFSA), has assessed whether the addition of the bacterial strain Bifidobacterium breve (*B. breve*) M-16V to infant formula could pose a health risk in the short or long term. VKM has evaluated the formula as full diet or supplementary diet for children aged 0-12 months.

Bifidobacteria are naturally found in the human gut and in breast milk. *B. breve* M-16V was originally isolated from the gut of an infant in the 1960s and is patented by the Japanese company Morinaga Milk, which distributes the bacterial strain to other manufacturers.

VKM has been asked to assess the addition of *B. breve* M-16V to infant formula in general, as well as in two specific products intended for infants with cow's milk allergy or other food allergies. The assignment from the NFSA is based on an application to market the products in Norway. The products in question are regulated as foods for special medical purposes. Live bacteria should not be added to foods if they may pose a health risk, and infants are considered a vulnerable group of the population.

Methods: VKM developed a protocol for the risk assessment based on guidelines for the assessment of probiotics in food from the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2001) and from VKM (2014), as well as general risk assessment methodology.

The sources of information used in the health risk assessment include underlying documentation for the regulatory status of microorganisms in the EU (QPS) and the USA (GRAS), as well as documentation from the applicant. Additionally, VKM has done a systematic review of clinical studies on infants conducted in countries where *B. breve* M-16V is permitted. VKM assessed the methodological quality of the studies and the confidence in the overall body of evidence for health outcomes of greatest clinical significance. Confidence was assessed on a scale from "very low" to "high" according to given criteria.

Basic genetic and phenotypic characteristics of *B. breve* M-16V were assessed based on laboratory studies and animal experiments described in GRAS reports, as well as additional documentation from the applicant. The characteristics include possible resistance to antimicrobial agents, production of pathogenic factors (virulence factors), toxins, and undesirable metabolites (D-lactate, biogenic amines, ammonia), some metabolic activities (azoreductase or nitroreductase activity), ability to hydrolyse bile salts, break down red blood cells (haemolysis), affect platelets, and adhere to mucous membranes and various cell types.

Possible health risks for Norwegian infants were assessed by compiling the basic characteristics of *B. breve* M-16V, results from VKM's review of published studies, and our own estimation of possible intakes of infant formula and *B. breve* M-16V.

Results: VKM finds it to be sufficiently documented that *B. breve* M-16V does not have unwanted genetic or phenotypic characteristics.

VKM included and quality-assessed 14 publications from 13 clinical studies involving a total of 1580 children who received infant formula with or without the addition of *B. breve* M-16V, 836 in test groups and 744 in control groups. Of the children who received *B. breve* M-16V, 491 were described as healthy, while 345 had cow's milk allergy or allergic skin symptoms. *B. breve* M-16V was administered in concentrations ranging from 1 × 10^6 to 1.47 × 10^9 colony-forming units (cfu) per 100 mL, often in combination with prebiotic fibres. The mixture of *B. breve* M-16V and prebiotics (synbiotic mixture) was given either as full diet (478 children) or as supplementary diet (342 children), and the interventions lasted from 6 weeks to 12 months.

VKM summarized reported side effects, growth disturbances, and various symptoms from the gastrointestinal tract, skin, and airways from the clinical studies. There were no consistent differences in the occurrence or type of side effects between the test and control groups in the studies. VKM found no growth disturbances as a result of consuming infant formula with *B. breve* M-16V, nor development or worsening of symptoms from the gastrointestinal tract, skin, or respiratory tract. The outcomes of side effects and growth were considered to have the greatest clinical significance, and VKM has moderate confidence in the overall body of evidence published to date for these outcomes.

VKM identified only two follow-up studies of children after the intervention period with *B. breve* M-16V had ended (long-term health effects). One study of infants with atopic dermatitis assessed the prevalence of asthma symptoms after one year, and one study of preterm infants receiving *B. breve* M-16V via tube feeding assessed neuropsychological outcomes after 3 to 5 years. The studies did not indicate negative effects of *B. breve* M-16V on these health outcomes.

Exposure: VKM has estimated that boys who receive infant formula as their sole nutrition at 12 months of age will have the highest intake of formula (theoretical maximum volume 14.5 dl/day) and therefore also of *B. breve* M-16V (2.13 × 10^9 cfu per day). VKM's calculation of infant formula as partial nutrition at 12 months shows an intake of *B. breve* M-16V in the range of 0.65-1.06 × 10^9 cfu per day (depending on the child's weight), which is comparable to the result from the literature search but based on only one study (0.79 × 10^9 cfu per day).

Uncertainty: The greatest uncertainty identified by VKM in the risk assessment is the possibility of undesirable long-term health effects of consuming *B. breve* M-16V during infancy and the mechanisms that could lead to such effects.

Conclusion: VKM finds no consistent differences in short-term health risks between the test and control groups in the studies of infant formula supplemented with *B. breve* M-16V. This applies to formula both as sole nutrition and as partial nutrition. However, there is some uncertainty associated with the conclusion, as VKM has "moderate confidence" in the evidence base for side effects and growth. "Moderate confidence" is due to the fact that most studies primarily aim to assess benefits rather than risks, and that manufacturers have contributed to the studies.

Knowledge gaps: VKM has found few studies that have investigated possible long-term effects of giving infant formulas with *B. breve* M-16V to infants. There is also a lack of clinical studies independent of the manufacturers. Furthermore, most clinical studies have aimed to investigate possible positive rather than negative health effects of *B. breve* M-16V.

Sammendrag på norsk

Bakgrunn: VKM har, på oppdrag fra Mattilsynet (MT), vurdert om tilsetning av bakteriestammen Bifidobacterium breve (*B. breve*) M-16V til morsmelkerstatning kan innebære helserisiko på kort eller lengre sikt. VKM har vurdert erstatning som eneste ernæring eller delernæring til barn 0-12 måneder.

Bifidobakterier finnes naturlig i tarmsystemet til mennesker og i morsmelk. *B. breve* M-16V ble opprinnelig isolert fra tarmen til et spedbarn på 1960-tallet, og er patentert av det japanske selskapet Morinaga Milk som distribuerer bakteriestammen til andre produsenter.

VKM er i oppdraget bedt om å vurdere tilsetning av *B. breve* M-16V i morsmelkerstatning generelt, samt i to spesifikke produkter beregnet for spedbarn med kumelkallergi eller andre matallergier. Oppdraget fra MT er basert på en søknad om å omsette produktene i Norge. Produktene det søkes om er regulert som næringsmidler til spesielle medisinske formål. Levende bakterier skal ikke tilsettes næringsmidler dersom det kan innebære helserisiko, og spedbarn regnes blant sårbare grupper av befolkningen.

Metoder: VKM utarbeidet en protokoll for risikovurderingen med utgangspunkt i retningslinjer for vurdering av probiotika i mat fra FNs matvareorganisasjon og Verdens helseorganisasjon (FAO/WHO, 2001) og fra VKM (2014), samt generell metode for risikovurdering.

Informasjonskildene som er brukt i vurderingen av helserisiko, er underliggende dokumentasjon for regulatorisk status for mikroorganismer i EU (QPS) og USA (GRAS), samt dokumentasjon fra søker. I tillegg har VKM gjort en systematisk kunnskapsoppsummering av kliniske studier på spedbarn utført i land hvor *B. breve* M-16V er tillatt. VKM har vurdert studienes metodiske kvalitet og tillit til samlet kunnskapsgrunnlag for helseutfall med størst klinisk betydning. Tillit vurderes på en skala fra «svært lav» til «høy» etter gitte kriterier.

Grunnleggende genetiske- og fenotypiske egenskaper ved *B. breve* M-16V er vurdert basert på laboratoriestudier og dyreforsøk beskrevet i GRAS rapporter, samt tilleggsdokumentasjon fra søker. Egenskapene innbefatter mulig resistens mot antimikrobielle midler, produksjon av sykdomsfremkallende faktorer (virulensfaktorer), toksiner og uønskede metabolitter (D-laktat, biogene aminer, ammoniakk), noen metabolske aktiviteter (azoreduktase- eller nitroreduktaseaktivitet), evne til å spalte gallesalter, bryte ned røde blodceller (hemolyse), påvirke blodplater, og binde seg til slimhinner og ulike celletyper.

Mulig helserisiko for norske spedbarn er vurdert ved å sammenstille de grunnleggende egenskapene til *B. breve* M-16V, resultater fra VKMs gjennomgang av publiserte studier og egen estimering av mulig inntak av morsmelkerstatning og *B. breve* M-16V.

Resultater: VKM mener det er tilstrekkelig dokumentert at *B. breve* M-16V ikke har uønskede genetiske- eller fenotypiske egenskaper.

VKM inkluderte og kvalitetsvurderte 14 publikasjoner fra 13 kliniske studier av til sammen 1580 barn som har fått morsmelkerstatning med eller uten tilsetning av *B. breve* M-16V, 836 i testgruppe og 744 i kontrollgruppe. Av barna som fikk *B. breve* M-16V, var 491 betegnet som friske, mens 345 hadde kumelkallergi eller allergiske hudsymptomer. *B. breve* M-16V ble gitt i konsentrasjoner fra 1×10^6 til 1.47×10^9 kolonidannende enheter (cfu) per 100 mL, ofte i kombinasjon med prebiotiske fibre. Blandingen med *B. breve* M-16V og prebiotika (synbiotisk blanding) ble gitt enten som fullernæring (478 barn) eller som delernæring (342 barn), og intervensjonene varte fra 6 uker til 12 måneder.

VKM har oppsummert rapporterte bivirkninger, forstyrrelser i vekstutvikling, og ulike symptomer fra mage- og tarm, hud og luftveier fra de kliniske studiene. Det var ingen konsistente forskjeller i forekomst eller type bivirkninger mellom test- og kontrollgruppe i studiene. VKM fant ingen forstyrrelser i vekstutvikling som følge av inntak av morsmelkerstatning med *B. breve* M-16V, heller ikke utvikling eller forverring av symptomer fra mage og tarm, hud eller luftveier. Utfallene bivirkninger og vekst ble tillagt størst klinisk betydning, og VKM har moderat tillit til det samlede kunnskapsgrunnlaget publisert per i dag for disse utfallene.

VKM identifiserte kun to oppfølgingsstudier av barn etter at intervensjonsperioden med *B. breve* M-16V var avsluttet (langsiktige helseeffekter). En studie av spedbarn med atopisk dermatitt vurderte forekomst av astma-symptomer etter ett år, og en studie av for tidlig fødte barn som fikk *B. breve* M-16V via sondeernæring vurderte nevropsykologiske utfall etter 3 til 5 år. Studiene viste ikke tegn til negative effekter av *B. breve* M-16V på disse helseutfallene.

Eksponering: VKM har estimert at gutter som får morsmelkerstatning som eneste ernæring ved alder 12 måneder, vil ha det høyeste inntaket av erstatning (teoretisk maksvolum 14.5 dl/dag) og derfor også av *B. breve* M-16V (2.13×10^9 cfu per dag). VKMs beregning av morsmelkerstatningen som delernæring ved 12 måneder, viser et inntak av *B. breve* M-16V i intervallet $0.65\text{-}1.06 \times 10^9$ cfu per dag (avhengig av barnets vekt) som er sammenlignetbart med resultat fra litteratursøket, men kun basert på én studie (0.79×10^9 cfu per dag).

Usikkerhet: Den største usikkerheten VKM har identifisert i risikovurderingen, er mulighet for uønskede langsiktige helseeffekter av inntak av *B. breve* M-16V i spedbarnstiden, og hvilke mekanismer som kan føre til slike effekter.

Konklusjon: VKM finner ingen konsistente forskjeller i helserisiko på kort sikt mellom test- og kontrollgruppene i studiene av morsmelkerstatning tilsatt *B. breve* M-16V. Dette gjelder erstatning både som eneste ernæring og som delernæring. Det er imidlertid noe usikkerhet knyttet til konklusjonen, da VKM har "moderat tillit" til kunnskapsgrunnlaget for bivirkninger og vekst. "Moderat tillit" skyldes at de fleste studiene har som hovedformål å se på nytte fremfor risiko, og at produsentene har bidratt i studiene.

Kunnskapshull: VKM har funnet få studier som har undersøkt mulige langtidseffekter av å gi morsmelkerstatninger med *B. breve* M-16V til spedbarn. Det er også mangel på kliniske studier som er uavhengig av produsentene. Videre har de fleste kliniske studiene hatt som formål å undersøke mulige positive framfor negative helseeffekter ved *B. breve* M-16V.

Abbreviations and glossary

Abbreviations

AAF – amino acid formula
AAF-Syn – amino acid formula with synbiotics
AMR – antimicrobial resistance
B. breve – Bifidobacterium breve
cfu – colony forming unit
EFSA – European Food Safety Authority
eHF – extensively hydrolysed formula
eHF-Syn – extensively hydrolysed formula with synbiotics
EU – European Union
FAO – Food and Agriculture Organization of the United Nations
FDA – U.S. Food and Drug Administration
GRAS – Generally Recognized As Safe
ITT – Intention-to-treat analysis
lcFOS – Long-chain fructo-oligosaccharides
LBW – low birth weight
LOS – late onset sepsis
NEC – necrotizing enterocolitis
NFSA – Norwegian Food Safety Authority
pAOS pectin-derived acidic oligosaccharide
PP – Per-protocol analysis
QPS – Qualified Presumption of Safety
RCT – Randomized controlled trial
RoB – Risk of bias
scGOS – short-chain galacto-oligosaccharides
SCORAD – SCORing Atopic Dermatitis (SCORAD) rating scale
SLR – systematic literature review
TFF – time to full enteral feed
ToR – terms of reference
VKM – Norwegian Scientific Committee for Food and Environment
WHO – World Health Organization

Glossary

Adverse event	Term for an unfavourable or harmful outcome that occurs during, or after, the use of a drug or other intervention, but is not necessarily caused by it (Peryer et al., 2024).
Adverse effect (or harm)	Term for an adverse event for which the causal relation between the intervention and the event is at least a reasonable possibility (Peryer et al., 2024).
BLAST	The Basic Local Alignment Search Tool (BLAST) finds regions of similarity between sequences. The program compares nucleotide- or protein sequences and calculates the statistical significance of matches (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
BLASTn	Nucleotide BLAST: compares one or more nucleotide query sequences to a subject nucleotide sequence or a database of nucleotide sequences. This is useful when trying to determine the evolutionary relationships among different organisms.
BLASTp	Protein BLAST: compares one or more protein query sequences to a subject protein sequence or a database of protein sequences. This is useful when trying to identify a protein
Colony forming unit	A unit of measurement used to determine the number of bacterial cells in a sample.
Colonization: transient, permanent	Colonization means infection and is the first stage of microbial infection by the establishment of the pathogen at the appropriate portal of entry. All multicellular organisms are colonized to some degree by extrinsic organisms, and the vast majority of these exist in either a mutualistic or commensal relationship with the host. The difference between an infection and colonization is often only a matter of circumstance. Non-pathogenic organisms can become pathogenic given specific conditions, and even the most virulent organism requires certain circumstances to cause a compromising infection (Dani, 2014). See also definition of infection.
Complementary foods	The first solid and semi-solid foods given to infants when human milk or infant formula is no longer sufficient to meet nutritional needs.
Generally Recognized As Safe (GRAS)	GRAS is a designation by the U.S. Food and Drug Administration (FDA) that a chemical or substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements.
Hypoallergenic	Do not contain allergens, and/or unlikely to cause allergic reaction.
Infant	Child younger than 12 months.

ITT population	In a randomized, controlled trial, the intention-to-treat (ITT) population represents all the study subjects who were randomized to the different treatment groups.
Microbiome	The microbiome is the collection of all microbes (e.g. bacteria, fungi, viruses) and their genes and biomolecules that are part of a given environment.
Microbiota	Collective term for microbial community (i.e., any type of microorganism) that may be found within a given environment.
PP population	In a randomized, controlled trial, the per-protocol population is defined as a subset of the intention-to-treat (ITT) population who completed the study without any major protocol violations.
Prebiotics	A prebiotic is a selectively fermented food ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Gibson et al., 2010)
Premature infant	Infant born before 37 weeks gestation.
Probiotics	Live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001).
Qualified Presumption of Safety	The QPS approach is a system similar in concept and purpose to the GRAS definition used in the USA but has been modified to take account of the different regulatory practices in Europe. With respect to probiotics, QPS represents a possible route to harmonisation of approaches for the safety assessment of microorganisms used in feed/food production.
Synbiotics	The term synbiotic is used for a product that contains both probiotics and prebiotics. Since the word alludes to synergism, this term should be reserved for products in which the prebiotic compound selectively favours the probiotic component.
Virulence factor	Virulence factors are microbe-associated molecules that are required for a microbe to cause disease while infecting eukaryotic hosts such as humans.

Background as provided by the Norwegian Food Safety Authority

The Norwegian Scientific Committee for Food and Environment (VKM) has previously published several benefit and risk assessments of bacterial strains with probiotic effect in products for infants and toddlers.

In these reports, VKM concludes, among other things, that long-term intake of the relevant bacteria in monoculture for the youngest age groups (those under 12 months) may have unknown health effects. Furthermore, the lowest age groups, i.e., four to six months and six to 12 months, are in the most immature and vulnerable phase when it comes to establishing bacterial flora in the intestine and developing the immune system. It will therefore be those age groups with the highest risk of any negative health effects from daily intake of probiotics.

All foods sold on the Norwegian market shall, pursuant to Section 16 of the Food Law, be safe. According to the preparatory works of the Law, stricter safety requirements are imposed on food products aimed to vulnerable groups in the population, such as infants and young children. There are also requirements in the legislation that these foods must be suitable for the relevant group of children. Food for special medical purposes is regulated by Regulation of 10 January 2014 No. 21 on foods for specific groups implementing Regulation (EU) 2016/128 into Norwegian law. Hypoallergenic nutritional products aimed at infants with, for example cow's milk allergy, fall under the provisions of these regulations. In these regulations, infants are defined as children under twelve months of age and toddlers are defined as children of one to three years of age.

A business operator wishes to market two products containing the bacterial strain *Bifidobacterium breve* M-16V on the Norwegian market. The products are defined as “for the dietary management of cow's milk allergy” and are registered as foods for special medical purposes. The business operator points out that both products are marketed in many countries in Europe. The business operator has submitted documentation about the bacterial strain, in addition to documentation that they believe supports that the products are safe and suitable for use in products intended for infants.

In light of the requirements regarding safety and suitability, the Norwegian Food Safety Authority requests VKM for a risk assessment of infant formula and supplement mixture with *Bifidobacterium breve* M-16V.

Considering the requirements regarding safety and suitability, the Norwegian Food Safety Authority requests VKM for a risk assessment of *Bifidobacterium breve* M-16V when added to infant formula in general, and when added to two specific hypoallergenic formula.

Terms of reference as provided by the Norwegian Food Safety Authority

The Norwegian Food Safety Authority asks VKM to answer the following questions:

1. Are there any health risks of giving infant formulas containing *Bifidobacterium breve* M-16V to infants 0-12 months:
    a. As a full diet
    b. As a supplementary diet
2. Are there any health risks of giving two specific hypoallergenic infant formulas containing *Bifidobacterium breve* M-16V (marketed as foods for special medical purposes) to infants with cow milk allergy and/or other food allergies and related allergic conditions:
    a. PEPTI SYNEO™ as full diet from 0-6 months
    b. PEPTI SYNEO™ as supplementary diet from 6-12 months
    c. NEOCATE SYNEO™ as full diet from 0-12 months

1 Introduction

1.1 Interpretation of the terms of reference

The terms of reference (ToR) have been interpreted by the VKM project group as follows:

- ToR 1: a request for a general scientific risk assessment of the safety of oral intake of *B. breve* M-16V through infant formula. The formula may be for general use, or special medical purposes (e.g., hypoallergenic), and may or may not contain prebiotics.
- ToR 2: a request for a risk assessment of two specific symbiotic products in an application to the NFSA. *B. breve* M16-V is added with prebiotics to a hypoallergenic infant formula for the management of cow's milk allergy and other allergic conditions.

The characteristics describing the safety of the *B. breve* M-16V bacteria will be common to both ToRs.

1.2 Limitation/delimitations

Common to ToR 1 and ToR 2

- Infants are defined as children aged 0 to 12 months as in the terms of reference from the NFSA.
- The risk assessment is performed for *B. breve* M-16V as monoculture, not in probiotic mixtures with other strains, but *B. breve* M-16V alone in symbiotic mixtures will be included.
- Potential beneficial effects of *B. breve* M-16V are not part of the mandate and have not been evaluated.
- Mechanistic evidence related to colonization of the host gastrointestinal mucosal surface (transiently or permanently), microbiome modulation and /or immune imprinting by *B. breve* M-16V has not been evaluated.
- Contamination during the manufacturing process of the *B. breve* M-16V strain or infant formula containing *B. breve* M-16V has not been evaluated.
- The health risks are limited to effects of *B. breve* M-16V, and not the risk of using liquid formula as a full diet for prolonged periods (e.g., potential effects on oral motor skills or speech-language development).

General risk assessment (ToR 1)

- The general risk assessment is performed for oral or enteral intake of *B. breve* M-16V for infants of any gestational age.

Product specific risk assessment (ToR2)

- The product specific health risks of two hypoallergenic infant formula containing *B. breve* M16-V are assessed for intended use and under the assumption that the products are prepared and used according to the manufacturer's instructions. Use is reported to be contraindicated in premature infants or infants who are immunocompromised.
- It is assumed that hypoallergenic infant formula for cow's milk allergy is used for children with clinical allergy who do not receive any human milk. The manufacturer has declared that the recommended use of the products is after full consideration of all feeding options, including breastfeeding with maternal elimination diet.
- Cow milk, cow milk products or human milk are not considered as complementary foods for children receiving hypoallergenic infant formula due to cow's milk allergy.

1.3 Introduction to probiotics

A widely adopted definition of "probiotics" is "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001; Hill et al., 2014). Probiotics (bacteria, yeasts) can be administered via different routes (oral, intravaginal, or topical). In this assessment, the term "probiotics" is used to describe any food-grade commercial microorganism. Commercial probiotics can be used as ingredients in functional foods, infant formula, and porridge, and can be taken or administered as a dietary supplement. Within the EU, the term "probiotic" on a food label requires an authorized health claim, but so far, no health claim has been approved for probiotic-containing foods (source: EU Register of Health Claims).

Many probiotics have regulatory status as safe, such as GRAS (generally regarded as safe) status in the United States, and QPS (Qualified Presumption of Safety) status in the European Union. The safety profile of probiotics in foods is mainly based on the history of safe use, and on observations noted in clinical trials assessing probiotics efficacy as the main outcome, rather than safety (Bafeta et al., 2018; Suez et al., 2019).

Several major mechanisms have been suggested to be involved in the functions of probiotics (Suez et al., 2019). These mechanisms include immunomodulation (through effects on expression of immune-related genes, inflammatory pathway activity and immune marker levels), direct and indirect pathogen antagonism, improved barrier function, and other mechanisms such as the ability to deconjugate bile acid, degrade lactose and complex carbohydrates, affect signalling to the enteric and central nervous systems, and modulate the indigenous microbiome. The mechanisms of action of probiotics are not completely understood and some remain debated, including the capacity of the administered microorganisms to colonize the host gastrointestinal mucosal surface (stably or transiently), and the degree of interaction with the host microbiome (Suez et al., 2019). Not all probiotics are thought to function in the same manner. Strain-specificity of probiotic effects is an important cornerstone principle of probiotic science, although certain mechanisms (Sanders et al., 2018) and genomic traits (Rodriguez and Martiny, 2020) may be shared between groups of bacteria at the species, or genus level.

1.4 Use of probiotics in infants

Probiotics are administered to infants to manage allergic conditions (e.g., cow's milk allergy or atopic dermatitis), and to preterm infants, in attempts to reduce neonatal morbidities such as necrotizing enterocolitis (NEC) and late-onset sepsis (van den Akker et al., 2020). In healthy infants, the purpose of giving probiotics may be to relieve gastrointestinal symptoms, and to reduce risk of infectious diseases and antimicrobial use (Indrio et al., 2022).

Differences in the gut microbiome of breast-fed and formula-fed infants, has motivated food manufacturers to add probiotics to infant formula, to compensate for the bacteria transmitted through breast feeding. Infants are considered a vulnerable group when assessing potential health risks of probiotics. In newborn children, a microbiota has not yet been established. Establishment of the GI microbiome is considered an essential developmental process, and is influenced by multiple factors, including maternal factors, host genetics, mode of delivery, gestational age, type of infant feeding (breastfeeding or formula feeding), antibiotics use, and environmental factors (Tamburini et al., 2016; Milani et al., 2017). The relative importance of these factors remains debated and reported influence on the microbiome is inconsistent.

It has been hypothesized that in infants, probiotic microorganisms could become primary colonizers that persist in the gut, perhaps even for life (FAO/WHO, 2001), with largely unknown health consequences. Disruptions in the development of the gut microbiome have been linked to health issues later in life, such as asthma and allergies, irritable bowel disease, overweight/obesity, and diabetes (Tamburini et al., 2016), although a role of probiotic infant formula in this process has not been established.

2 Methodology and data

A study protocol with an outline of methods and data sources was developed by the project group and assessed by the approval group prior to the assessment. The protocol is available as a supplementary file on the report's webpage at VKM.no.

The general risk assessment follows the steps: (i) hazard identification and hazard characterization, (ii) exposure assessment, and (iii) risk characterization.

Infant formulas in general are regulated as foods for specific groups, and hypoallergenic formulas as foods for special medical purposes. Therefore, risk is assessed based on the FAO/WHO guideline for the evaluation of probiotics in foods (FAO/WHO, 2001) stating that no adverse effects related to probiotic administration should be experienced when food is considered. Further, bacteria which contain transmissible drug resistance genes, should not be used in foods. Evaluations of the safety of probiotics should include studies in humans and be based on intended use of products.

2.1 Information sources

VKM has relied on the following sources of information:

a. Documentation published by the U.S. Food and Drug Administration (FDA) in GRAS Notice No. 453, 454, and 455 on *B. breve* M-16V (FDA, 2013a; FDA, 2013b; FDA, 2013c) with special emphasis on No. 455 for intended use as an ingredient in exempt term powdered amino acid-based formula *B. breve* M-16V.
b. Documentation from the applicant according to VKM's Guidelines for assessment of safety aspects of probiotic (food) products (VKM, 2014) and an additional file with clinical documentation.
c. The most recent qualified presumption of safety (QPS) status of microorganisms intentionally added to food or feed as notified to EFSA (2024b)
d. Systematic literature review of human studies in infants performed by VKM (methods described in section 2.2 and results in section 3.12
e. Input data for exposure calculations in Norwegian infants: national recommendations on infant nutrition, the national guideline for newborn medicine, the WHO (2006) child growth standard, and a systematic review to estimate intake for formula during complimentary feeding (as specified in section 4)

In the general risk assessment, characteristics of the *B. breve* M-16V bacteria based on in vitro and animal studies (listed under section 3.1) were assessed based on sources a) GRAS Notices and b) documentation from the applicant. There was a high degree of overlap between documentation from the applicant in Norway and what the company used to achieve GRAS status in the US. Therefore, VKM has emphasized documentation in GRAS assessments, although GRAS status does not apply in Europe. Regarding human studies of *B. breve* M-16V in infants, GRAS Notices No. 453 to 455 were all prepared in 2012, and no search strategy was reported in either a) or b). Therefore, VKM decided to do an independent systematic literature search for studies on health effects, as described below (section 2.2.). The search was supplemented with screening of studies cited in sources a) or b).

In VKM's calculations of exposure to *B. breve* M-16V through infant formula intake in Norwegian infants (section 4), data were also used from Information regarding VKM's guideline and check list. The applicant, along with their documentation, submitted their responses to the checklist in VKM's guidelines for evaluating of probiotic product. For more information regarding this, see Appendix 10-1.

The product specific assessment was based on the general risk assessment and information sources a) to e) limited to sub-populations of infants with allergic conditions.

2.2 Systematic literature review of health risks

As part of the general risk assessment, VKM conducted a systematic literature review (SLR) of the evidence of health risks (short term or long term) in relation to the probiotic strain *B. breve* M-16V when given in infant formula, orally or enterally (by nasogastric tube). The SLR was conducted according to a protocol described within the overall study protocol. The SLR included literature search and selection of publications according to predefined eligibility criteria, data extraction, a risk of bias assessment (RoB) of the included publications, and a qualitative evidence synthesis. The SLR is reported in accordance with the PRISMA guideline for reporting of systematic reviews (Page et al., 2021). The different steps are described in more detail below.

2.2.1 Eligibility criteria

Eligibility criteria are summarized below in Table 2.2.1-1. Human studies with all common study designs except cross-sectional, were eligible for inclusion. The study population was infants aged 0-12 months in accordance with the ToR. Intake should be oral or enteral. Probiotics are widely administered enterally for complications in premature infants who often have low birth weight (LBW). VKM considered studies of *B. breve* M-16V in these infants as relevant to inform on potential health risks and eligible for inclusion, but as supportive evidence. Under the assumption of strain specificity, studies of *B. breve* M-16V in mixture with other probiotic bacteria were ineligible. There were no restrictions on dose or duration of intake or the type of intervention (risk reduction, prevention, treatment, or symptom relief). Studies should have a comparator without *B. breve* M-16V unless the outcome was probiotic sepsis for which no comparator was considered necessary. There were few restrictions placed on outcomes a priori, except some considered to have uncertain clinical relevance (faecal microbiota composition, stool characteristics, changes in markers of immunomodulation and inflammation, and bacteremia without sepsis). Studies of beneficial effects were eligible for inclusion to study the possibility of unintended effects such as adverse events or worsening of conditions or symptoms for which *B. breve* M-16V was administered. Both review studies and primary studies were eligible for inclusion without time or language restrictions. More details on the rationale for some of the eligibility criteria can be found in the study protocol.

Table 2.2.1-1. Overview of eligibility criteria for human studies in general risk assessment.

Criterion	Eligible for inclusion	Excluded
Study design	Case reports of adverse events, randomized controlled trials (RCTs), and observational studies with confounder control	Cross-sectional studies
Population	Infants aged 0-12 months, including vulnerable groups (e.g. premature, or low birth weight infants)	Infants treated medically with immunosuppression or with chemotherapy, or treated surgically for intestinal/digestive conditions
Intervention or exposure	Intake (oral or enteral) of *B. breve* M-16V as monoculture, alone or in synbiotic mixtures, in infant formula or as supplement or medical use	Probiotic mixtures with other strains, prebiotics only, postbiotics
Comparator	Infant feeding (oral or enteral) without probiotics, may contain prebiotics	
Outcome	Outcomes measured or reported in relation to safety or health risks, short term, or long term.	Outcomes with unclear health risk (changes in faecal microbiota composition, stool characteristics, markers of immunomodulation and inflammation, C-reactive protein), bacteremia without sepsis
Language	No restrictions.	Studies that cannot be machine translated, if required
Date	No restrictions (inception to search date).	
Type of literature	Previous review studies, systematic or non-systematic, primary studies in peer-reviewed journals, grey literature.	Reports (editorials, book chapters, comments, letters) without original data, and conference abstracts

2.2.2 Search strategy

The six databases Ovid MEDLINE, Ovid Embase, Web of Science, Cochrane Central Register of Controlled Trials (CENTRAL), Cochrane Database of Systematic Reviews, and Epistemonikos were searched from inception to August 23 (MEDLINE) or August 24 (other databases) in 2023 in collaboration with a research librarian. The full search strategy is provided in Appendix 10.1.

The following information sources were also searched for additional studies:

1. GRAS Notice No. 455 (Table 11. Human Clinical Studies of *Bifidobacterium breve* — Infants. Publications from 1992 to 2012)
2. Clinical documentation provided by the applicant as part of the application to the NFSA
3. Review studies and overview of review studies, identified in VKM's database search.
4. A report from the Scientific Committee of the Food Safety Authority of Ireland (FSAI) on the "Assessment of the safety of "probiotics" in food supplements" (section 1.6 on Reported adverse events associated with the consumption of "probiotics") which was published in July 2024 (FSAI, 2024).

2.2.3 Publication selection

Pairs of members from the project group independently screened all records from VKM's search against eligibility criteria. The screening was done in two steps: first by 1) titles or abstracts using Rayyan software for blinding, and then 2) as full text records in EndNote. Discrepancies were resolved by consensus.

In the full text screening stage, publications without original data (e.g., comments, editorials), were read to identify any additional studies before they were excluded. Abstracts in conference proceedings were also excluded, but attempts were made to identify a subsequent journal publication. Multiple publications of the same study were excluded to avoid data duplication. Older versions of updated Cochrane reviews were excluded. Reviews of probiotics without summarized results on *B. Breve* M16-V were excluded, but references to primary studies on *B. Breve* M-16V were checked against VKM's search.

To avoid duplication of previous literature reviews, VKM followed a two-step approach where review studies were evaluated first. The need to summarize primary studies was determined based on the topics covered, risk of bias, and search dates in the review studies. Thus, some reviews and primary studies were included for detailed evaluation and some data extraction but excluded from the final evidence synthesis.

Based on the identified review studies and risk of bias evaluation (section 3.12.3), VKM decided to do an independent literature review of studies on *B. breve* M-16V in infant formula but rely on systematic reviews for *B. breve* M-16V in enteral feeding of premature and/or low birth weight infants. The reviews were supplemented with any primary studies on enteral feeding from VKM's search that were published after the reviews.

2.2.4 Data extraction and synthesis – review studies

Data were extracted from the studies by one reviewer. Data were extracted to different templates created in Excel so that similar information was extracted from all studies. One template was created for basic information about each review that included the type of review, overall study objective, protocol registration, funding source, author affiliations (e.g. hospital, university, industry), databases searched, search date, eligible study designs, eligible publication types, and the PICO or PECO elements (study population, intervention or exposure, comparator, and outcomes). Some additional information to assess risk of bias using the ROBIS tool (section 2.2.5) was extracted directly to the ROIBS forms.

When review studies presented multiple health outcomes, VKM extracted results on outcomes compatible with the VKM's eligibility criteria. For each outcome, the number of studies on *B. breve* M-16V, study designs, number of participants, dose range, and author conclusion were extracted. If the outcome was meta-analysed, the pooled estimate with 95% confidence interval and p-value, type of model (fixed- or random effects), and measure of between study heterogeneity (I^2) were extracted, and the overall confidence rating of the evidence when given. Results were presented for each included review without any further synthesis of the results.

2.2.5 Risk of bias assessment – review studies

After full-text screening, VKM used the ROBIS tool (Whiting et al., 2016) to formally assess the internal validity, also referred to as risk of bias (RoB) of the included review studies. The tool is completed in three phases to 1) assess relevance (optional), 2) identify concerns with the review process based on four different domains and 3) judge the risk of bias. VKM assessed relevance based on how well the PICO elements (population, intervention/exposure, comparator, and outcomes) in the evaluated reviews matched VKM's eligibility criteria (see section 2.2.1).

The four domains of the review process (Phase 2) that may introduce bias are the study eligibility criteria, identification and selection of studies, data collection and study appraisal, and synthesis and findings. The last phase considers whether the systematic review overall is at risk of bias, and the final rating is either low, unclear, or high.

2.2.6 Data extraction and synthesis – primary studies

Background information from the studies were extracted by one reviewer. Exposure data and results on health outcomes were extracted by one reviewer and checked by a second reviewer. The Excel template for background information included study objective, study design, intake of *B. breve* M-16V (enteral or formula), infant population (e.g. preterm, healthy, or allergic/atopic), study country/countries, inclusion period (calendar years), age of infants at inclusion, size of study samples (intention to treat and per protocol for RCTs), name of study (when given), clinical trial or other protocol registration, and industry affiliation (funding and/or authorship).

A separate template for formula exposure included details on the interventions or exposure, including the intervention arms in RCTs (e.g. synbiotic vs control), duration of intervention or exposure, concentration of *B. breve* M-16V, the content of prebiotics in synbiotic formula, the control group (e.g. test formula without synbiotic, or test formula with prebiotic), formula description (e.g. hydrolysed, or amino-acid formula), formula energy content, formula intake assessment (e.g. parent reported diaries for a certain number of days, or at different ages), feeding regimen (e.g. exclusive formula, or mixed feeding), and formula brand/supplier. In studies on enteral feeding, only information regarding dose on *B. breve* M-16V was extracted.

Another template for outcome data included all health outcomes identified within each publication, status of the outcome (primary, secondary, or a safety parameter), where the outcome result was located (text or table number) and if the outcome should be included or excluded according to VKM's eligibility criteria (section 2.3.1). For included outcomes, the method for the outcome assessment and results were extracted. Quantitative estimates were extracted when available. A narrative synthesis of the evidence was chosen due to the heterogenous result presentation among the included studies.

Some additional items were also extracted as part of the risk of bias assessment (section 2.2.7), such as information about the randomization process, allocation concealment, and blinding in RCT studies, confounder treatment in observational studies, and attrition during the study.

2.2.7 Risk of bias assessment – primary studies

After full-text screening, risk of bias (RoB) was evaluated using the OHAT (Office of Health Assessment and Translation) tool (OHAT, 2015; OHAT, 2019). Two reviewers independently assessed RoB for each included primary study on *B. breve* M-16V in infant formula or enteral feeding. The reviewers calibrated themselves to ensure similar evaluation.

The OHAT tool includes eight questions considering aspects relevant for RoB evaluation of human controlled trials, and seven questions for cohorts and other observational studies (Table 2.2.7-1):

Table 2.2.7-1. Overview of RoB questions evaluated for the RCTs and observational studies.

Bias domains and questions	RCTs	Cohorts
Selection bias		
1. Was administered dose or exposure level adequately randomized?	X	
2. Was allocation to study groups adequately concealed	X	
3. Did selection of study participants result in appropriate comparison groups?		x
Confounding bias		
4. Did the study design or analysis account for important confounding and modifying variables?		x
Performance bias		
6. Were the research personnel and human subjects blinded to the study group during the study?	x	
Attrition/exclusion bias		
7. Were outcome data complete without attrition or exclusion from analysis?	x	x
Detection bias		
8. Can we be confident in the exposure characterization?	x	x
9. Can we be confident in the outcome assessment?	x	x
Selective reporting bias		
10. Were all measured outcomes reported?	x	x
Performance bias		
11. Were there no other potential threats to internal validity (e.g., industry affiliation and authorship and statistical methods were appropriate)?	x	x

The response options and symbols (in parentheses) used for the rating are i) definitely low risk of bias (++); ii) probably low risk of bias (+); iii) probably high risk of bias/not reported (NR) (-); and iv) definitely high risk of bias (- -).

The project group defined blinding (question 6), exposure characterization (question 8), and outcome assessment (question 9) as key questions for the RCTs. The key questions address the elements performance bias (blinding of personnel and participants) and detection bias (confidence in the exposure characterization, and the outcome assessment). For observational studies we defined questions on confounding (question 4), as well as questions 8, and 9 as key questions, addressing confounding bias, and detection bias. The non-key questions address the elements selection bias, attrition/exclusion bias, selective reporting bias, and “other” sources of bias.

The rating of key and non-key questions was integrated to classify the RCTs into tiers to characterize the overall RoB for each outcome/study as shown in Table 2.2.7-2. Tier 1 represents low RoB; Tier 3 represents high RoB. Tier 2 studies are all studies not meeting the criteria for Tier 1 or Tier 3.

In accordance with the OHAT guidance document, the criteria for three questions should be explicitly customized for each evaluation: 1) consideration of potential confounders, 2) confidence in the exposure characterization, and 3) confidence in the outcome assessment. Different outcomes within the same publication were scored separately. Thus, the same study may end up in different tiers of RoB, depending on the outcome.

Details on the customized criteria for exposure and outcomes are presented in Appendix 10.6. According to OHAT, questions about "other" sources of bias can also be customized. For the current risk assessment VKM evaluated statistical methods, co-exposures (e.g., complimentary feeding, prebiotics, or antibiotic use in studies of formula) and industry affiliation/co-authorship. When the question of "other" bias covers multiple sources, the source with the lowest score will determine the overall score for the question.

Table 2.2.7-2. Classification of studies into tiers according to overall RoB for each outcome/study.

Tier	1	2	3
Criteria for classification	Definitely low, or probably low RoB for key items AND Definitely low, or probably low RoB for most other applicable criteria	All combinations not falling under tier 1 or 3	Definitely high, or probably high RoB for key items AND Definitely high, or probably high RoB for most other applicable criteria

2.2.8 Confidence ratings and evidence of health risk – review and primary studies

For systematic review studies, VKM used the study author conclusions regarding the overall confidence in the body of evidence, if provided.

For primary studies, VKM generated evidence profiles and confidence ratings according the OHAT approach (OHAT, 2019). The overall confidence rating in the body of evidence for an association between an intervention/exposure and a specific health outcome can be "high," "moderate," "low" or "very low or no evidence identified." When no health effect or health risk is identified, the ratings describe the confidence in the body of evidence that the intervention or exposure is not associated with the health outcome.

High Confidence (++++): the true effect is highly likely to be reflected in the apparent relationship. Further research is very unlikely to change confidence in the apparent relationship.

Moderate confidence (+++): the true effect may be reflected in the apparent relationship.

Low Confidence (++): the true effect may be different from the apparent relationship.

Very Low Confidence (+): the true effect is highly likely to be different from the apparent relationship. Further research is very likely to have an impact on confidence in the apparent relationship.

To arrive at the final confidence rating, available studies on a particular outcome are first grouped by key study design features and given an initial confidence rating (from high to very low). This initial rating can then be downgraded for factors that decrease confidence in the results (risk of bias, unexplained inconsistency, indirectness or lack of applicability, imprecision, and publication bias) and upgraded for factors that increase confidence in the results (large magnitude of effect, dose response, consistency across study designs/populations, and consideration of residual confounding or other factors that increase our confidence in the association or effect).

The conclusion for the level of evidence for a particular health effect (here health risk) is reached by combining the final confidence rating in the association between exposure to the substance ("high," "moderate," "low," or "very low" confidence) as described above, with the nature of the effect ("health effect" or "no health effect"). The five descriptors used to categorize the level of evidence for the health effect are "high," "moderate," "low," "evidence of no health effect," and "inadequate evidence". If the nature of the effect is «no health effect», the confidence in the evidence must be «high» to conclude that there is evidence of no health effect. For other confidence levels, the evidence is considered insufficient (Table 2.2.8-1). This is due to the inherent difficulty in proving a negative, or no association (OHAT, 2019).

Table 2.2.8-1. Translation of certainty in body of evidence to level of evidence for health effect (OHAT, 2019).

Confidence in the body of evidence	Level of evidence for health effect/ no health effect	Definition
Health effect		
High	High	There is high confidence in the body of evidence for an association between exposure to the substance and the health outcome(s).
Moderate	Moderate	There is moderate confidence in the body of evidence for an association between exposure to the substance and the health outcome(s).
Low	Low	There is low confidence in the body of evidence for an association between exposure to the substance and health outcome(s), or no data are available.
Very low or no evidence identified	Inadequate	There is insufficient evidence available to assess if the exposure to the substance is associated with the health outcome(s).
No health effect		
High	Evidence of no health effect	There is high confidence in the body of evidence that exposure to the substance is not associated with the health outcome(s).
Moderate	Inadequate	There is insufficient evidence available to assess if the exposure to the substance is associated with the health outcome(s).
Low	Inadequate	There is insufficient evidence available to assess if the exposure to the substance is associated with the health outcome(s).
Very low or no evidence identified	Inadequate	There is insufficient evidence available to assess if the exposure to the substance is associated with the health outcome(s).

2.3 Use of artificial intelligence (AI)

The VKM project group has used AI for language translations in the current risk assessment. One publication (Hattori et al., 2003) was translated from Japanese to English using Google Lens. The publication was later excluded from the final evidence synthesis based on the translated text. The project group wrote the summary section in Norwegian and used Microsoft 365 Copilot for Enterprise with data protection for translation into English. The translated version was checked and modified by the group.

3 Hazard identification and characterization of *B. breve* M-16V

3.1 *B. breve* M-16V

The genus Bifidobacteria is a non-sporulating, anaerobic, non-motile, Gram-positive Y-shaped bacterium which lives symbiotically in the intestines of humans and is found in breast milk.

Bifidobacterium Breve (B. breve) M-16V is a commercial strain of bacteria owned and distributed by the Japanese company Morinaga Milk Industry Co., Ltd. It was isolated from the faeces of a healthy infant in 1963 and has been available in Japan since 1976. Other species names include *B. breve* Reuter 1963 AL, Mitsuoka 16, PRSF-B105, BB-576, biovar a, YY, and Yaeshima M-16V (FDA, 2013c)

The strain was originally deposited with the Belgian Co-ordinated Collections of Microorganisms (BCCM) with LMG accession number 23729 but has been moved to the NITE Patent Microorganisms Depositary (NPMD) at the National Institute of Technology and Evaluation (NITE) in Japan with accession number NITE BP-02622. VKM has received a certificate of deposition of *B. breve* M-16V at NPMD but has not been able to locate the deposition on the open website.

In this risk assessment, the following phenotypic, genetic, and genomic properties related to the safety of *B. breve* M-16V have been assessed: antimicrobial resistance patterns and whether resistance genes are located on, or associated with mobile genetic elements (e.g. plasmids, transposons or integrons); virulence factors; specific metabolic activities (production of D-lactate, biogenic amines, ammonia production, bile salt deconjugation, azoreductase or nitroreductase activity); haemolytic activity; effects on platelet aggregation or viability; adherence to mucus and/or human epithelial cells and cell lines; and toxin production. In addition, health risks of *B. breve* M-16V when used in enteral feeding or infant formula have been assessed based on human studies in infants.

3.2 Regulatory status in the EU: QPS

The concept of “qualified presumption of safety” (QPS) was introduced by EFSA to facilitate a harmonized generic pre-assessment of microorganisms to support safety risk assessments of regulated products, including food additives (EFSA, 2007). The QPS list (“Updated list of QPS-recommended microorganisms for safety risk assessments carried out by EFSA”) is a “positive list” containing only the taxonomic units notified to EFSA for which QPS status has been granted. The QPS approach considers the taxonomic identity, the body of relevant knowledge, safety concerns and occurrence of antimicrobial resistance.

The list of QPS-recommended microorganisms is reviewed every 6 months by the EFSA Panel on Biological Hazards (BIOHAZ) following a literature search for publicly available studies reporting on safety concerns for humans, animals or the environment caused by organisms that have QPS status (EFSA et al., 2024b).

For bacteria, QPS status is granted at the species level, and the species *B. breve* has QPS-status according to the most recent version of the QPS list (version 20) from July 2024 (EFSA et al., 2024a). As of January 2022, the updated list is only provided as an Excel file, which was checked by VKM.

3.3 Regulatory status in the US: GRAS

B. breve M-16V was determined to be generally regarded as safe (GRAS) for intended use as an addition to powdered amino acid-based exempt term infant formulas, including powdered amino acid-based exempt term infant formulas for the management of allergies in infants in GRAS Notice No. 455 (FDA, 2013c). The concentration and exposure scenario in the GRAS dossier is described in more detail in section 4.4. The GRAS Notice was prepared for Danone Trading B.V.

The following sections 3.4-3.11 are largely a summary of the evaluation of documentation in GRAS Notice No. 455 on phenotypic, genetic, and genomic properties of *B. breve* M-16V, as well as results from safety studies on animals. All references in these sections refer to the GRAS report and not the current report. More details of the GRAS-documentation are available in Appendix 10-3. The GRAS documentation has been supplemented with any additional information from the applicant.

Section 3.12 summarizes VKM's systematic literature review of studies of *B. breve* M-16V in infants, including studies cited in GRAS Notice No. 455 (publications until 2012).

3.4 Antimicrobial resistance patterns

Based on *in vitro* studies included in the GRAS report, *B. breve* M-16V did not exhibit concerning levels of antimicrobial resistance, and no plasmids were found in the strain. No information from the applicant was provided regarding other types of mobile genetic elements (e.g., transposons, integrons or bacteriophages). Since no AMR genes were identified on the genome, this information seems of little relevance to hazard evaluation.

As it has been stated by the applicant, the absence of antimicrobial resistance genes in *B. breve* M-16V was determined by searching for genomic sequences with homology to known antibiotic resistance genes found in other strains of bifidobacteria and lactobacilli (Appendix 6 in GRAS Notice No. 455). The information in appendix 6 in GRAS is insufficient and the applicant did not provide more information, as it was requested by VKM. The information provided regarding antimicrobial resistance properties of *B. breve* M16V is considered sufficient (see data gaps section). The risk associated with transfer of AMR genes from *B. breve* M16V to other bacterial species is considered minimal.

3.5 Virulence factors

Bioinformatic analysis of the *B. breve* M-16V genome did not indicate significant pathogenic potential.

The applicant has provided some information regarding pathogenicity of *B. breve* M-16V, although the source of the database is not clearly defined in the GRAS documentation. Generally, the strain is not considered pathogenic, although some cases of bacteremia and septicaemia have been reported in the immune-compromised patients. In vivo toxicological studies did not indicate any abnormal effects. A series of phenotypic tests also indicated an absence of cytotoxic properties (see next section). From the provided documentation, as well as the existing literature, there is no real indication that *B. breve* M-16V acts as a pathogen.

3.6 Specific metabolic activities

The metabolic activities considered were production of D-lactate, biogenic amines, ammonium production, bile salt deconjugation, azoreductase or nitro reductase activity.

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for these characteristics of *B. breve* M-16V. For detailed GRAS information, see Appendix 10-3.

3.7 Haemolytic activity

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for this characteristic of *B. breve* M-16V. For detailed GRAS information, see Appendix 10-3.

3.8 Effects on platelet aggregation or viability

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for this characteristic of *B. breve* M-16V. For detailed GRAS information, see Appendix 10-3.

3.9 Adherence to mucus and/or human epithelial cells and cell lines

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for this characteristic of *B. breve* M-16V. For detailed GRAS information, see Appendix 10-3.

3.10 Toxin production

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for this characteristic of *B. breve* M-16V. For detailed GRAS information, see Appendix 10-3.

The information provided in sections 3.4-3.10, which is from GRAS evaluation of *B. breve* M-16V, was evaluated by the project group. The applicant has supplied further information via the checklist provided within VKM's Guidelines for assessment of safety aspects of probiotic (food) products (VKM, 2014). Information regarding production of D-lactate, biogenic amines, ammonia production, azoreductase or nitro reductase activity, effects on platelet aggregation or viability is considered sufficient. However, the project group had supplementary questions to the applicant regarding haemolytic activity, bile salt deconjugation, adherence to mucus and/or human epithelial cells and cell lines but did not receive more information from the applicant. However, the information contained within the GRAS dossier, checklist, and additional documentation can be considered acceptable.

3.11 Acute, subacute, and chronic animal toxicity

VKM has evaluated the GRAS documentation, and the applicant did not provide any additional information. Based on the available documentation, VKM agrees with the GRAS conclusion for this characteristic of *B. breve* M-16V. For detailed GRAS information, see Appendix 10.3.

Based on the information provided by the applicant in the GRAS, the documentation regarding acute, subacute, and chronic animal toxicity after oral administration of the *B. breve* M16-V is considered sufficient. The project group did not have supplementary questions for the applicant regarding these issues.

3.12 Human studies of infant health risk

This section summarizes the results of VKM's systematic literature review of studies in infants receiving B. breve M-16V in infant formula or as enteral feeding (see section 2.2 for the methods description). The study selection, study characteristics, results of VKM's risk of bias evaluation, and the study results are presented in separate sections below, first for formula studies, then for enteral feeding as supporting evidence.

3.12.1 Study selection – results

The study selection is summarized in the PRISMA flow chart (Figure 3.12.1-1).

The literature search identified 1513 records from the six research databases after duplicate removal. Of 1513 records, 200 were screened as full text. VKM included five reviews and 24 primary publications (15 on formula feeding and nine on enteral feeding) with strain specific results on B. breve M-16V for further evaluation, including study overlap (between reviews and primary publications, and between multiple primary publications from the same study) and the risk of bias.

The five review studies evaluated by VKM were Athalye-Jape et al., 2018; Kulkarni et al., 2022; Sorensen et al., 2021b; van den Akker et al., 2018; Wong et al., 2019.

The 15 publications on formula feeding were Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Chatchatee et al., 2022; Chua et al., 2017; Fox et al., 2019; Harvey et al., 2014; Hattori et al., 2003; Hulshof et al., 2018; Phavichitr et al., 2021; Sorensen et al., 2021a; Taniuchi et al., 2005; van der Aa et al., 2010; van der Aa et al., 2011; Wang et al., 2021 The nine publications on enteral feeding of preterm infants were: Agrawal et al., 2020; Athalye-Jape et al., 2020; Umezaki et al., 2010; Inage et al., 2022; Patole et al., 2014; Patole et al., 2016a; Patole et al., 2016b; Priyadarshi et al., 2021; Wang et al., 2007.

Among the 15 publications on formula feeding, VKM identified three follow-up publications: Taniuchi et al., 2005 had longer follow-up for atopic dermatitis (three months) than Hattori et al., 2003 (one month) but presented results for the full intervention period (0-3 months) with minor differences in study sample. Therefore, Hattori et al., 2003 was excluded. Fox et al., 2019 was a follow-up of Candy et al., 2018 (ASSIGN study) and also presented results for the full intervention period (0-26 weeks vs. 0-8 weeks in Candy et al., 2018), but with some variations in the result presentation. Both publications from the ASSIGN study were therefore kept. Van der Aa et al., 2011 was a follow-up of the study sample in van der Aa et al., 2010, but presented a different health outcome, and both were kept. Of note, Harvey et al., 2014 presented results from two different studies within the same publication (referred to as study 1 in healthy children, and study 2 in children with cow's milk allergy). Thus, 14 publications from 13 different studies were included as evidence on formula feeding.

Of the nine publications on enteral feeding of preterm infants, only the four most recent were included (Agrawal et al., 2020; Athalye-Jape et al., 2020; Inage et al., 2022; Priyadarshi et al., 2021) to supplement two included review studies on enteral feeding (Athalye-Jape et al., 2018; Kulkarni et al., 2022). The other three reviews were excluded (described in section 3.12.3).

After manually searching additional information sources (described in section 2.2.2), VKM identified and screened six reviews/reports and six primary publications. No additional publications were included. The six primary publications were found by searching GRAS Notice No. 455 (FDA, 2013c) and a review by Wong et al., 2019 (described in section 3.12.2), which included Japanese databases. All publications described infants receiving enteral nutrition or in intensive care. Of note, two were published under a different strain name (*B. breve* BB-576 or *B. Breve* biovar a). Three of six publications, all in Japanese journals, seemed to fulfil the eligible criteria based on the English abstracts or English summaries in the GRAS report. However, these older studies were difficult to retrieve as full texts (cited in FDA, 2013c: Yamada et al., 2002; Sato et al., 2003 and cited in Wong et al.,2019: Hiroaki et al., 2009) and were not evaluated further as VKM considered studies of enteral feeding as supportive evidence and had decided to rely on systematic reviews supplemented with more recent primary studies from VKM's search.

A list of all publications screened, and reasons for exclusions of all full-text publications, is available as a supplementary file to the risk assessment.

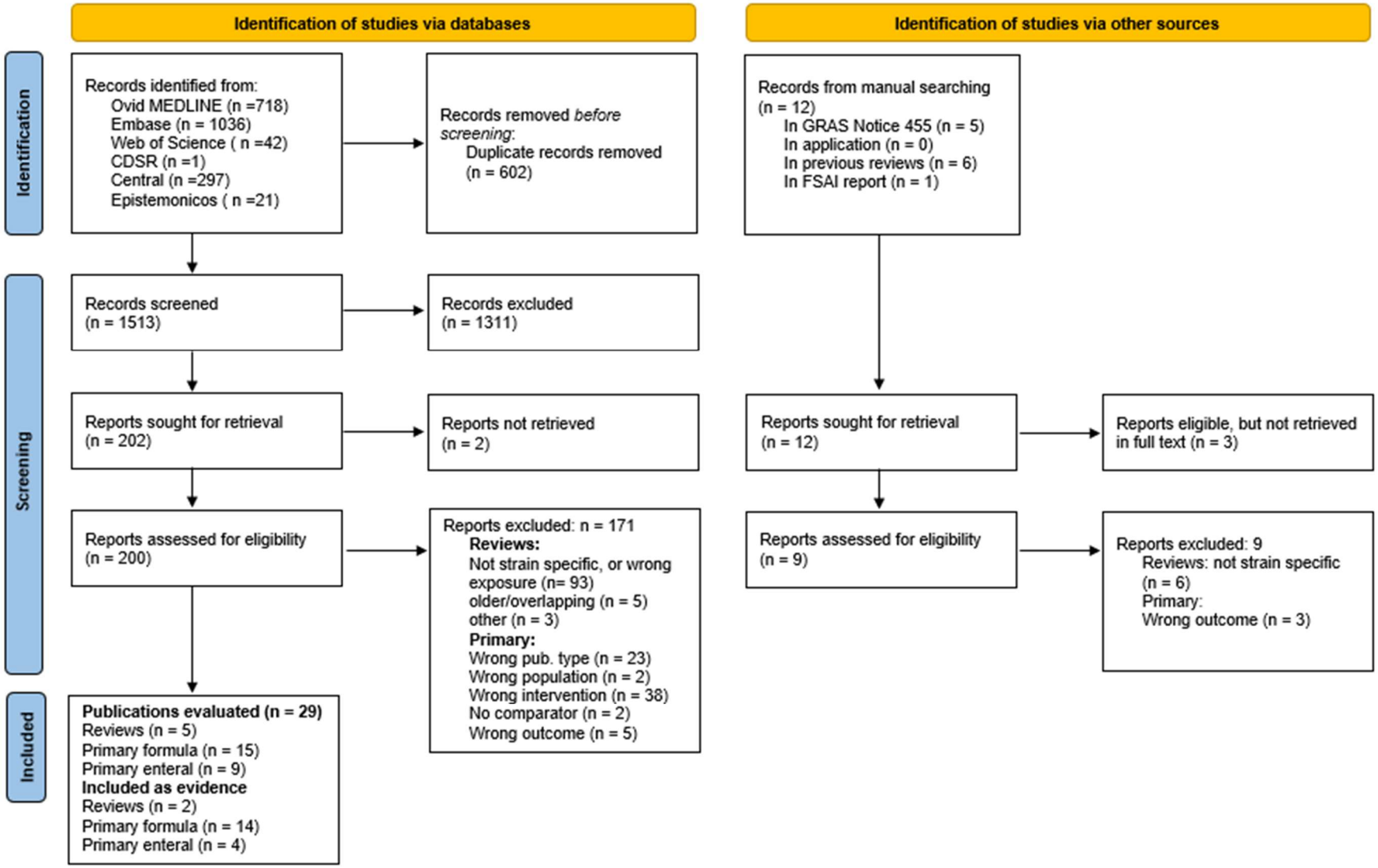

Figure 3.12.1-1. PRISMA flow chart of the study selection

3.12.2 Review studies – characteristics

Details of the five review studies evaluated by VKM are presented in Table 3.12.2-1. The reviews were published from 2018 to 2022 (Athalye-Jape et al., 2018; Kulkarni et al., 2022; Sorensen et al., 2021b; van den Akker et al., 2018; Wong et al., 2019). Two of five reviews focused on enteral feeding of premature- or low birth weight infants and the effects of probiotics or B. breve M-16V on related birth complications such as necrotizing enterocolitis and late-onset sepsis (Athalye-Jape et al., 2018; van den Akker et al., 2018). One additional review in preterm infants focused exclusively on reports of probiotics sepsis (Kulkarni et al., 2022). Of note, this review had null findings on B. breve M-16V, which was considered a result, and the publication was therefore evaluated. Of the remaining reviews, one focused on hypoallergenic infant formula for infants with cow's milk protein allergy and a range of clinical outcomes (Sorensen et al., 2021b), and one covered B. breve M-16V as a probiotic or synbiotic for either premature birth complications or allergic disorders (Wong et al., 2019). Two of five reviews were funded by or affiliated with the manufacturer of formula (Sorensen et al., 2021b) or the M-16V strain (Wong et al., 2019). Wong et al., 2019 was the only review that reported to have searched Japanese databases, which was considered relevant as B. breve M-16V is manufactured by a Japanese company (Morinaga Milk). All review studies were assessed for risk of bias (Section 3.12.3).

As the only systematic review identified on formula feeding was restricted to infants with cow's milk allergy (Sorensen et al., 2021b), and the risk of bias was graded "high" (see section 3.12.3), VKM decided to do an independent literature review of formula feeding.

Table 3.12.2-1: Characteristics of evaluated review studies with strain-specific results on *B. breve* M-16V in infants

Author, year	Type of review	Protocol registration	Objective	Databases searched	Search date	Publications on B. breve M-16V	Funding source
Athalye-Jape et al., 2018	Systematic review (strain specific) and meta-analysis	Not reported	To conduct a SLR of studies of *B. breve* M-16V in preterm infants	CENTRAL, MEDLINE, Ovid EMBASE, Ovid CINAHL, Google Scholar, databases for grey literature	Inception to 5 Jan 2017	9 (5 RCTs, 4 non-RCTs), 6 in meta-analysis	Not reported, no financial disclosures
Kulkarni et al., 2022	Systematic review (probiotic sepsis) no meta-analysis	Not reported	To conduct a SLR for reports of probiotic sepsis in preterm infants	CENTRAL, PubMed, Ovid EMBASE, Ovid CINAHL, Google Scholar, databases for grey literature, trial registries	Inception to Jan 2022	No studies identified	Not reported
Sorensen et al., 2021b	Systematic review (hypoallergenic infant formula with synbiotics) and meta-analysis	Not reported	To conduct a SLR to examine whether hypoallergenic formulae containing synbiotics could have a beneficial effect on clinical outcomes in infants with CMPA.	MEDLINE, EMBASE, Cochrane Library	Inception to November 2020	7 (5 papers, 2 abstracts) on 4 RCTs, 3 in meta-analysis	Nutricia Ltd. No external funding.
van den Akker et al., 2018	Systematic review (probiotics grouped by strain) and network meta-analysis	PROSPERO (ID=CRD42017064847)	To determine the most effective probiotic strain in reducing mortality and morbidity in preterm infants	PubMed	Inception to 19 Sept 2017	4 RCTs, all in meta-analysis	Unrestricted ESPGHAN[1] grant
Wong et al., 2019	Review (strain specific) no meta-analysis	Not reported	To discuss the effects of probiotic administration on infant health, with specific attention to the probiotic strain M-16V	MEDLINE, EMBASE, ICHUSHI web (Japanese) and JDreamIII (Japanese)	Inception to 12 May 2019	1 review and 13 original papers in infants (single strain or synbiotic), 11 on preterm birth complications, 3 on allergenicity, 0 on safety	Morinaga Milk Industry Co., Ltd. No external funding.

[1]ESPGHAN=European Society for Paediatric Gastroenterology Hepatology and Nutrition.

3.12.3 Review studies – risk of bias

The ROBIS tool (described in section 2.2.5) was used to screen for relevance and assess the risk of bias (Table 3.12.3-1) for each review study. Relevance was determined based on how well the review being evaluated matched the eligibility criteria set by VKM (section 2.2.1). Relevance was considered "partial" for all reviews, meaning that not all, but some results, matched VKM's target questions.

Based on the risk of bias evaluation, two reviews graded "low risk of bias" were included (Athalye-Jape et al., 2018; Kulkarni et al., 2022) in the risk assessment, the remaining were excluded but screened for any primary studies not identified by VKM. Both included reviews assessed enteral feeding of premature infants. VKM identified four additional primary studies on enteral feeding with *B. Breve* M-16V published after the search dates in the included reviews. The primary studies are described in section 3.12.4 and assessed for risk of bias with the OHAT tool for primary studies in section 3.12.5.

The synthesis of the screening with the ROBIS tool is given in Table 3.12.3-1. Kulkarni et al., 2022 reported null results for reports of probiotic sepsis related to *B. breve* M-16V. The overall ROBIS grade was therefore based on the first two domains only (study eligibility and identification of studies), as the last two domains (data collection and synthesis) became non-applicable (n/a). The detailed ROBIS scoring sheets are available as supplementary files to this risk assessment.

Table 3.12.3-1: ROBIS profile table of 5 evaluated systematic reviews.

Author, year	Phase 2				Phase 3
	1. Study eligibility criteria	2. Identification and selection of studies	3. Data collection and study appraisal	4. Synthesis and findings	RISK OF BIAS IN THE REVIEW
Athalye-Jape et al., 2018	Low	Low	Unclear	High	Low
Kulkarni et al., 2022	Low	Low	n/a[1]	n/a[1]	Low
Sorensen et al., 2021b	High	High	High	Unclear	High
van den Akker et al., 2018	High	High	Unclear	High	High
Wong et al., 2019	High	High	High	High	High
High	3	3	2	3	3
Low	2	2	0	0	2
Unclear	0	0	2	1	0

[1]n/a, not applicable due to no identified studies (null results) in Domain 2.

Table 3.12.3-2: Overview of relevance evaluation for reviews with strain-specific results on *B. breve* M-16V in infants.

Author, year	Study populations	Intervention	Comparator	Outcome groups	Relevance/ROBIS screening	ROBIS risk of bias
Included						
Athalye-Jape et al., 2018	Preterm neonates, gestational age <37 weeks or low birth weight (<2500 g)	Enteral administration, any dose of *B. breve* M-16V commenced within the first 10 days of life, continued for at least 7 days	Placebo or control	Premature birth complications and faecal microbiota	Partial, some outcomes (birth complications)	Low
Kulkarni et al., 2022	Preterm infants	Enteral administration of probiotic or synbiotic in any strain, dose, or duration.	Not specified/not applicable	Probiotic sepsis	Partial (null results on *B. breve* M-16V)	Low
Excluded						
Sorensen et al., 2021b	Infants and children aged <3 years with IgE or non-IgE mediated CMPA	Any amino acid formula with synbiotics (AAF-Syn) or extensively hydrolysed formula (eHF) with synbiotics (eHF-Syn)	Unrestricted	Clinical symptoms and allergenicity	Partial (results in infants)	High
van den Akker et al., 2018	Preterm infants, gestational age <37 weeks	Probiotic treatment against placebo, usual care, or head-to-head with a different probiotic regime	Probiotic treatment as only difference	Premature birth complications	Partial (results on *B. breve* M-16V)	High
Wong et al., 2019	Animal (mice, rat) or human (infant, children, adults)	Probiotic administration of *B. breve* M-16V (single strain, probiotic mixture, or as synbiotic)	Not specified	Premature birth complications, allergic disorders, safety/stability, gut microbiota, other	Partial (human studies in infants, single strain only, some outcomes)	High

3.12.4 Primary studies – characteristics

Within the current section, studies on formula feeding are described first (study characteristics in Table 3.12.4-1 and exposure characteristics Table 3.12.4-2) before studies on enteral feeding (study characteristics in Table 3.12.4-3).

There were 14 publications from 13 different studies on formula feeding, 12 with an RCT design and one observational (Sorensen et al., 2021a). The study samples were counted once for studies with a follow-up publication. The 13 studies comprised 1580 infants in total who were given test formula with *B. breve* M-16V (n=836) or control formula without *B. breve* M-16V (n=744). The total sample size per study ranged from 15 to 247 subjects. Age of the infants at inclusion ranged from birth to 3 years of age, but few children where older than 12 months. The subjects were recruited from 2003 to 2020. Of 13 studies, five were performed in Europe, three in USA, four in Asia and one multicentre study had participating countries from Europe and Asia in addition to the USA. Five studies (n=914) were performed in healthy infants (Abrahamse-Berkeveld et al., 2016; Harvey et al., 2014; Phavichitr et al., 2021; Wang et al., 2021) including one study in infants delivered by caesarean section (Chua et al., 2017), and eight studies (n=666) in infants with allergic disorders; six studies in infants with cow´s milk allergy (Burks et al., 2015; Chatchatee et al., 2022; Fox et al., 2019; Harvey et al., 2014; Sorensen et al., 2021a; Taniuchi et al., 2005) and two studies in infants with atopic dermatitis (Hulshof et al., 2018; van der Aa et al., 2011).

Most RCTs compared formula with synbiotics to control formula without synbiotics (two parallel groups). Only three studies had a design where the effect of *B. breve* M-16V could be separated from that of prebiotics, either because the RCT had a separate intervention arm for prebiotics (Chua et al., 2017), or because the prebiotic was part of the control formula (Taniuchi et al., 2005; Wang et al., 2021), see further descriptions of exposure in Table 3.12.4-2.

Table 3.12.4-1: Overview of 14 included and one excluded publication (13 primary studies) on infant formula with strain-specific results on *B. breve* M-16V.

Author, year	Study country	Inclusion years	Indication	Age at inclusion	Study sample	Design	Clinical trial or other protocol registration
Abrahamse-Berkeveld et al., 2016	Germany	2005-2006	Healthy	< 35 days (5 weeks)	228 infants randomized to test (105) or control (123) formula. Analysis of 100/110 (ITT) or 45/57 (PP)	RCT (2 parallel groups)	International Standard Randomised Controlled Trial Number: ISRCTN23993517
Burks et al., 2015	USA	2008-2012	Cow's milk allergy	0–8 months	110 infants randomized to test (54) or control (56) formula. Analysis of 54/56 (ITT) or 43/47 (PP/completers)	RCT (2 parallel groups), multicentre	ClinicalTrials.gov: NCT00664768
Candy et al., 2018/Fox et al., 2019	UK, Italy, Belgium, Sweden	2013-2015	Cow's milk allergy	< 13 months	71 infants randomized to test (35) or control (36 formula). Analysis of 35/36 (ITT) or 28/32 (PP)	RCT (2 parallel groups), multicentre	Netherlands Trial Register: NTR3979
Chatchatee et al., 2022	Germany, Italy, Singapore, Thailand, UK, USA	2013-2017	Cow's milk allergy	≤13 months	169 infants randomized to test (80) or control (89) formula. Analysis of all-subjects randomized or all-subjects treated (safety) but equal samples.	RCT (2 parallel groups), multicentre	Netherlands Trial Register: NTR3725
Chua et al., 2017	Singapore, Thailand	2011-2013	Healthy/caesarean delivery	Birth (1–3 days at the latest)	153 infants randomized to synbiotic (52), prebiotic (51) or control (50) formula. Analysed as modified intention-to-treat (stool samples), or PP populations, or All Subjects Treated (safety)	RCT (3 parallel groups), multicentre, exploratory	Netherlands Trial Register: NTR2838
Harvey et al., 2014 (study 1)	USA	2008-2011	Healthy	Birth to 15 days	115 infants randomized to test (59) or control (56). Analysis of 59 /56 (ITT) or 45/38 (PP growth) or 58/54 (PP stool and GI symptoms)	RCT (2 parallel groups)	ClinicalTrials.gov: NCT00664768 (study 2). No registration number found for study 1.
Harvey et al., 2014 (study 2)	USA	Not found	Cow's milk allergy	Birth to 3 yrs	30 infants aged birth to 3 yrs	RCT (2 cross-over groups), followed by open challenge	ClinicalTrials.gov: NCT00664768
Hulshof et al., 2018	Nether-lands	2012-2014	Atopic dermatitis with elevated Ig-E levels	0-11 months	31 infants randomized to test (16) or control (15) formula. Analysis of 13/13 (PP)	RCT (2 parallel groups)	Netherlands Trial Resister: NTR3447
Phavichitr et al., 2021	Thailand	2013-2015	Healthy	43 to 65 days (6 to 9 weeks)	247 infants randomized to test1 (81), test 2 (82) or control (84) formula. Analysis of all (ITT) or 78/78/83 (PP)	RCT (3 parallel groups), exploratory	ClinicalTrials.gov: NCT01813175

Author, year	Study country	Inclusion years	Indication	Age at inclusion	Study sample	Design	Clinical trial or other protocol registration
Sorensen et al., 2021a	UK	2020	Cow's milk allergy	Diagnosis ≤12 months age, mean age 4.7 months	148 infants prescribed synbiotic (74) or control (74) formula. Infants matched 1:1 for age at diagnosis, sex and observation period.	Retrospective cohort	THIN database (protocol reference number: 20-009, 12 August 2020)
Taniuchi et al., 2005	Japan	Not found	Cow's milk hypersensitivity with atopic dermatitis, and faecal microflora containing less than 30% Bifidobacterium	3.1 to 18.5 months	17 randomized to addition to formula (10) or no addition (7)	RCT (2 parallel groups), but two different doses in intervention group	Not reported
van der Aa et al., 2010/van der Aa et al., 2011	Nether-lands	2005-2007	Atopic dermatitis	< 7 months	90 infants randomized to test (46) or control (44) formula. Analysis of 42/43 (PP)	RCT (2 parallel groups), multicentre	International Standard Randomised Controlled Trial Number: ISRCTN69085979
Wang et al., 2021	China	2018-2019	Healthy	≤ 44 days (6 weeks)	224 infants randomized to test (112) or control (112) formula. Analysis of 89 /97 (PP at 17 weeks)	RCT (2 parallel groups), multicentre	ClinicalTrials.gov: NCT03520764
Excluded							
Hattori et al., 2003 (see Taniuchi et al., 2005)	Japan	Not found	Cow's milk hypersensitivity with atopic dermatitis, and faecal microflora containing less than 30% Bifidobacterium	3.1 to 18.5 months	15 infants randomized to probiotic addition to formula (8) or no addition (7)	RCT (2 parallel groups), but two different doses in intervention group	Not reported

An overview of exposure characteristics is given in Table 3.12.4-2.

Fox et al., 2019 and Candy et al., 2018 reported on different exposures and follow-up times; Fox et al. on the full 26 weeks of intervention and Candy et al., 2018 until week 8 when some study subjects were switched to a different formula "appropriate for their condition and age per clinicians' choice and practice". Study product was continued by 86% and 92% of test and control subjects between week 8–12, and by 71% and 80%, respectively until week 26. Thus, when Fox et al., 2019 reports results for the full 26 weeks, formula exposure differes from the original test/control formula in Candy et al., 2018, for some subjects.

In 13 studies, all infant formulas were provided by Nutricia (n=11) or Morinaga Milk Industries (n=2) and consisted of a prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS). The prebiotic formula served as base for adding a specific concentration of *B. breve* M-16V. The two studies with the three-armed intervention design compared a standard non-hydrolysed cow milk-based formula with both synbiotic and prebiotic formula (Chua et al., 2017) or a prebiotic formula with two different concentrations of *B. breve* M-16V (Phavichitr et al., 2021). Otherwise, the control formulas were either extensively hydrolysed (63 % of proteins <1000 Da) whey protein-based powder products or amino-acid based formulas. All formulas were isoenergetic with 66-67 kcal (276 kJ) energy per 100 mL and otherwise nutritionally complete. Three studies did not provide any information about the feeding regimen (Burks et al., 2015, Sorensen et al., 2021a, Taniuchi et al., 2005). Three studies specifically described that the infants were exclusively formula fed, meaning that they were not given any other food but the study formula (Harvey et al. 2014; Phavichitr et al., 2021; Wang et al., 2021). Two studies described that the infants were given formula on demand (Abrahamse-Berkeveld et al., 2016; van der Aa et al., 2010) and VKM interpreted that these infants were exclusively formula fed.

The infant formulas contained *B. breve* M-16V in a concentration range of 1×10^6 to 1.47×10^9 colony forming units (cfu) per 100 mL and were given to the test groups as full diet in 478 infants or supplementary diet in 358 infants, for an intervention period of six weeks to 12 months (median 16 weeks).

Three of the studies specified the volume of formula intake at different ages of the subjects (Abrahamse-Berkeveld et al., 2016, Candy et al., 2018, Chatchatee et al., 2022). Abrahamse-Berkeveld, M, et al. 2016 reported mean intake of 795-680 mL per day of formula in infants of 3-46 months. Candy et al. described that the infants were given a minimum of age-specific, daily formula from end of week 2 (age 0-6 months, 500 mL; 6-8 months, 450 mL; >9 months, 350ml). After eight weeks most of the infants in Candy et al., continued with the study formula or received a prescribed formula appropriate for their condition and age per clinicians' choice and practice (Fox et al., 2019). Chua et al., 2018 described that study formulas were administered from birth until 16 weeks of age as mixed feeding with breastfeeding. Chatchatee et al., 2022 reported an intake of 602 and 538 mL per day in infants of 6 and 12 months, respectively.

Ten human studies comprised 283 infants with cow´s milk allergy or hypersensitivity and 62 infants with atopic dermatitis. The study formulas were manufactured and provided by Nutricia in nine of ten studies. Morinaga Milk industries provided the infant formula in one study. The concentration of *B. breve* M-16V ranged from 7.5×10^8 to 1.47×10^9 cfu per 100 mL. The age of the infants at inclusion was from birth to three years, and the duration of the intervention ranged from 8 weeks to 12 months. The study formula was given as a full diet in 46 infants and as a supplementary diet in 299 infants.

Table 3.12.4-2. Overview of exposure in 14 included publications (13 primary studies) on infant formula containing *B. breve* M-16V

Author, year	Intervention arms (duration)	B. breve M-16V	Prebiotic	Control	Formula description	Feeding regimen
Abrahamse-Berkeveld et al., 2016	Synbiotic vs control (13 wks)	1.3×10^9 cfu/100 mL	Mixture of scGOS/lcFOS[1] in a 9:1 ratio (0.8 g per 100 mL formula)	Test formula without synbiotic	Extensively hydrolysed (63 % of proteins <1000 Da) whey protein-based powder	Ad libitum exclusively with allocated formulas
Burks et al., 2015	Synbiotic vs control (16 wks)	1.47×10^9 cfu/100 mL	Chicory-derived neutral oligofructose, long-chain inulin (BENEO-Orafti SA, Oreye, Belgium) and pectin-derived acidic oligosaccharide (pAOS) with a total amount of 8 g/l (6.8 g/l oligofructose: inulin 9:1 and 1.2 g/l pAOS).	Test formula without synbiotic	Amino-acid formula Neocate Infant DHA and ARA (SHS International Ltd., Nutricia Advanced Medical Nutrition, Liverpool, UK)	No description found
Candy et al., 2018	Synbiotic vs control (8 wks)	1.47×10^9 cfu/100 mL	Chicory-derived neutral oligofructose and long-chain inulin (0.63g/100ml)	Test formula without synbiotic	Amino-acid formula (Neocate LCP; Nutricia Advanced Medical Nutrition, Liverpool, UK)	A minimum, age-specific, daily formula intake from end of week 2 (age 0-6 months, 500 mL; 6-8 months, 450 mL; >9m, 350ml)
Fox et al., 2019 with subjects from Candy et al., 2018						After 8 weeks, subjects received a prescribed formula appropriate for their condition and age per clinicians' choice and practice.
Chatchatee et al., 2022	Synbiotic vs control (12 months)	1.47×10^9 cfu/100 mL	Chicory-derived neutral oligofructose and long-chain inulin (BENEO-Orafti SA, Oreye, Belgium; 9:1 ratio at a total concentration of 0.63 g/100 mL)	Test formula without synbiotic	Amino-acid formula	A minimum, age-specific, daily study product intake: 0 to 8 months, 450 mL; 9 to 18 months, 350 mL; and > 18 months, 250 mL

Author, year	Intervention arms (duration)	B. breve M-16V	Prebiotic	Control	Formula description	Feeding regimen
Chua et al., 2017	Synbiotic vs prebiotic vs control (16 wks)	7.5×10^8 cfu/100 mL	0.8 g/100 mL scGOS/lcFOS[1]	Test formula without synbiotic	Non-hydrolysed cow milk-based formula (Danone Nutricia Research, the Netherlands)	Study formulas administered from birth until 16 weeks of age, mixed feeding with breastfeeding
Harvey et al., 2014 - study 1	Synbiotic vs control (16 wks)	1.47×10^9 cfu/100 mL (10^8 cfu/g powder)	Inulin derived neutral oligofructose (Beneo P95 Raftilose P95) (Beneo Orafti S.A., Oreye, Belgium) and long-chain inulin (Beneo HP Raftiline HP) (Beneo Orafti S.A.) 9:1 ratio, and a pectin-derived acidic oligosaccharides (OS). Total amount of OS 8 g/l with 6.8 g/l neutral OS (85 weight %), and 1.2 g/l pectin-derived acidic OS (15 weight %).	Test formula without synbiotic. Medium chain triglyceride concentration test and control 33 vs 4 g/100 g total fatty acids (FA), and alternative source of essential FA (17.5% fat as low erucic acid rapeseed oil, canola oil instead of soy oil).	Neo (Nutricia, SHS International, Liverpool, UK) or Neo-Syn (Nutricia, SHS International).	Exclusive formula feeding
Harvey et al., 2014 – study 2	Synbiotic vs control, crossover (duration unknown) + 7-day open challenge	As above	As above	As above	As above	Formula challenge, followed by 7-day open challenge
Hulshof et al., 2018	Synbiotic vs control (4 months)	10^8 cfu/g powder (1.38×10^9 cfu/100 mL)[2]	0.8g/100 mL scGOS/lcFOS[1] (9:1)	Test formula without synbiotic	Extensively hydrolysed whey-based infant formula (Nutricia, Cuijk, the Netherlands)	Infants required to drink ≥500 mL/day of formula, partial breastfeeding allowed. Time (months) of first solid food introduction test (5.2) and control (5.3) groups
Phavichitr et al., 2021	Synbiotic vs control (6 wks)	1 × 10exp4 or 1×10^6 cfu/mL (1×10^6 or 1×10^8 cfu/100 mL)	0.8g/100 mL scGOS/lcFOS[1] (9:1)	Test formula with prebiotics scGOS/lcFOS (9:1), 0.8g/100 mL		Exclusive formula feeding

Author, year	Intervention arms (duration)	B. breve M-16V	Prebiotic	Control	Formula description	Feeding regimen
Sorensen et al., 2021a	Synbiotic vs control (mean (SD) duration on synbiotic formula 6.65 (5.3) months	Not given	Chicory-derived oligo-fructose and long-chain inulin	Test formula without synbiotic	Amino-acid formula	Unknown. Infants prescribed an AAF supplemented with *B. Breve* M16-V
Taniuchi et al., 2005	*B. breve* M16V (two doses) vs control (3 months)	5×10^9 or 15×10^9 cfu per day, fixed doses	Raffinose 0.13 g/100ml	Test formula with prebiotics	Extensively hydrolysed and ultrafiltrated bovine casein formula (New MA-1)	Not described
van der Aa et al., 2010/van der Aa et al., 2011	Synbiotic vs control (12 wks)	1.3×10^9 cfu/100 mL	Mixture of 90% scGOS and 10% lcFOS[1] (Immunofortis, Nutricia Cuijk B.V., Cuijk, the Netherlands), 0.8 g/100 mL	Test formula without synbiotic	Extensively hydrolysed whey-based formula (Nutrilon Peptis, Nutricia, Zoetermeer, the Netherlands)	Formula given on demand. Parents advised not to introduce solid foods before age 4–6 months
Wang et al., 2021	Synbiotic vs control with prebiotic (until age 17 wks)	3 × 107 cfu/g (4.1 × 108 cfu/100 mL)[2]	Mixture of 0.8 g/100 mL scGOS/lcFOS (9:1 ratio).	Intact cow's milk protein-based formula with prebiotics (Aptamil Pronutra).	Partially hydrolysed non-ultrafiltrated cow's milk protein-based formula with synbiotics. Test formula contained more carbohydrates (7.2 vs 6.7 g/100 mL) compensated by less fat (3.4 vs 3.5 g/100 mL) compared with control formula.	Exclusive formula feeding

[1]lcFOS, long-chain fructo-oligosaccharides, scGOS, short-chain galacto-oligosaccharides.

[2]When reported in grams of dry powder, VKM has estimated the concentration of *B. breve* M-16V in ready to eat formula using 13.8 g powder per 90 mL water to make 100 mL using the manufacturer's instructions for Aptamil Pepti SYNEO (4.6 g per 30 mL)

VKM identified four primary studies on enteral nutrition published after 2018 (Table 3.12.3-5). Three Australian and one Japanese study enrolled a total of 652 infants from 2010 to 2020 (Agrawal et al., 2020; Athalye-Jape et al., 2020; Inage et al., 2022; Priyadarshi et al., 2021). The study infants were preterm and/or had low or extremely low birth weight (<1000 g). One study was a follow-up (3 to 5 years) of 67 children from an RCT with 159 infants (Agrawal et al., 2020). The other three studies were treated as having an observational design (section 3.12.5): one study was nested within an RCT but used controls from a different trial (Athalye-Jape et al., 2020), and the remaining two were retrospective (Inage et al., 2022; Priyadarshi et al., 2021).

Table 3.12.3-5. Overview of four included publications (four primary studies) on enteral nutrition published after 2018.

Author, year	Study country	Inclusion years	Indication	Gestational age at inclusion (weeks)	Study sample	Design	Clinical trial or other protocol registration
Agrawal et al., 2020	Australia	2010-2012, follow-up 3 to 5 years	Preterm (<33 weeks gestation)	Median (IQR) 28 (26.0-29.9)	67 children (42%) of the 159 participants in the original trial, from test (36) or control (31) group	RCT, follow-up 3-5 years	Australia New Zealand Clinical Trial Registry ACTRN 12609000374268
Athalye-Jape et al., 2020	Australia	2015-2016	Preterm (<28 weeks gestation)	Median (IQR) 26.3 (25.2-26.9) for single-strain and 26.1 (25.2-26.9) in controls	154 infants from the SiMPro trial (single strain: 75 and three-strain: 79) and 29 infants from the placebo arm of the PANTS trial (controls)	Nested within RCT, controls from different trial	Australia New Zealand Clinical Trial Registry CTRN12615000940572
Inage et al., 2022	Japan	2015-2020	Extremely low birth weight (<1000 g)	Median (IQR) 26.8 (24.8-28.4)	55 infants, hyperglycemia 23 and non-hyperglycemia 32	Retrospective observational, medical records	Not found
Priyadarshi et al., 2021	Australia	2014-2018	Preterm (<32 weeks gestation and/or birth weight <1,500 g)	Mean (SD) 28.6 (± 2.27) in single strain and 28.3 ± 2.5 in two strain group	180 preterm infants in the two-strain, 196 in the single strain group from the two equal consecutive 2-year epochs	Retrospective observational	Sydney Children's Hospitals Network Human Research Ethics Committee: 2019/ETH09863, approved the study along with a waiver of consent

3.12.5 Primary studies – risk of bias

The OHAT tool (described in section 2.2.7) was used to classify primary studies into tiers describing the overall risk of bias (RoB). The RoB tiers were grouped by the health outcomes (Table 3.12.5-1) for which study results were extracted (sections 3.12.6 to 3.12.10). For infant formula studies, the health outcomes presented are (number of studies): adverse events (n=10), infant growth (n=10), gastrointestinal symptoms (n=8), skin symptoms (n=8), and airway symptoms (n=5). Most studies presented multiple health outcomes and could end up in different tiers for RoB depending on the outcome.

Table 3.12.5-1. Risk of bias (RoB) classification in tiers grouped by health outcomes in included studies on infant formula

	Tier for risk of bias		
Outcome (studies)	**1[1]**	**2[2]**	**3[3]**
Adverse events (n=10)	6	2	2
Growth indicators (n=9)	5	3	1
Gastrointestinal symptoms (n=8)	5	3	0
Skin symptoms (n=8)	5	2	1
Airway symptoms (n=5)	4	1	0

[1]Definitely low or probably low RoB for key items, and definitely low or probably low RoB for most other applicable criteria, see section 2.3.5.
[2]All combinations not falling under Tier 1 or 3.
[3]Definitely high or probably high RoB for key items, and probably or definitely high RoB for most other applicable criteria.

Most studies were in the Tier 1 category (low risk of bias). The publications by Candy et al., 2018 and Fox et al., 2019 (both ASSIGN study) were scored individually, but due to large overlap they received the same overall score and contributed as one study (Table 3.12.5-1). Several studies in Tier 2 or 3 did not report a method for how the outcome was assessed and therefore received a "probable high" risk of bias score for this key question, in accordance with the OHAT guideline for how to treat missing information. All studies on infant formula were authored or co-authored by the manufacturer or *B. Breve* M-16V or synbiotic formula and therefore received a "probable high" risk of bias for "other" sources of bias. But according to the scoring system, "other" sources were not a key question, and studies could still be categorised as Tier 1 overall. The scores for each RoB item for the different studies are presented in Appendix 10.4.

For studies on enteral feeding, the RoB tiers were also grouped by the health outcomes (Table 3.12.5-2) for which study results were extracted (section 3.12.12), but the four included studies all assessed different health outcomes: time to full enteral feeds (TFF), stage ≥ 2 necrotising enterocolitis (NEC), late onset sepsis (LOS), and infant growth indicators (n=1); probiotic sepsis (n=1); hyperglycemia (n=1), and neuropsychological development (n=1). The scores for each RoB item for the different studies are presented in Appendix 10.5.

Table 3.12.5-2. Risk of bias (RoB) classification in tiers grouped by health outcomes in included studies on enteral feeding

	Tier for risk of bias		
Outcome (studies)	**1[1]**	**2[2]**	**3[3]**
TFF[4], Stage ≥ 2 NEC[4], LOS[4], growth indicators (n=1)		1	
Neuropsychological development measures (n=1)	1	1	
Hyperglycemia (n=1)		1	
Probiotic sepsis, *B. Breve* M-16V (n=1)			1

[1]Definitely low or probably low RoB for key items and definitely low or probably low RoB for most other applicable criteria, see section 2.3.5.
[2]All combinations not falling under Tier 1 or 3.
[3]Definitely high or probably high RoB for key items and definitely high or probably high RoB for most other applicable criteria.
[4]TFF: time to full enteral feeds, NEC: necrotising enterocolitis, LOS: late onset sepsis.

3.12.6 Results formula feeding – adverse events (AE)

Of note, VKM adopted the definitions of "adverse events" (AE) and "adverse effects" used in the Cochrane Handbook for Systematic Reviews of Interventions (Peryer et al., 2024) stating that an adverse event is not considered an adverse effect unless there is at least a reasonable possibility of a causal relation between the intervention and the event. Thus, VKM has reported on the treatment relation described in the included studies.

Ten publications reported on AE (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Chatchatee et al., 2022; Chua et al., 2017; Fox et al., 2019; Harvey et al., 2014; Phavichitr et al., 2021; van der Aa et al., 2010; Wang et al., 2021) from ten studies, but not in a one-to-one relation. As previously described, Candy et al., 2018 and Fox et al. 2019 were overlapping (ASSIGN study) and Harvey et al., 2014 described two different studies. All studies had a RCT design. A total of 736 infants received *B. breve* M-16V in test formula and 648 received control formula (study descriptions in Table 3.12.4-1 and Table 3.12.4-2).

Most publications reported AE as "at least one" AE, or "any" AE, and/or the number of "serious" adverse event (SAE), and some reported dropout due to AE. However, definitions of AE and SAE were inconsistent, or lacking (four studies). An example of a wide definition of AE was: "any symptom or disease episode that occurred during the study" (van der Aa et al., 2010). An example of definition of SAE was: "any untoward medical occurrence resulting in death, is life-threatening (at the time of the event), requires hospitalisation or prolongation of existing hospitalisation or results in persistent or significantly disability or incapacity" (Abrahamse-Berkeveld et al., 2016). Fox et al. listed the three severe adverse events a posteriori as gastroesophageal reflux disease, laryngitis viral, and bronchiolitis that required hospitalization, and one anaphylactic reaction to pineapple. VKM also noted that number of SAEs was not always possible to separate from total AEs (Abrahamse-Berkeveld et al., 2016). In two studies there was an unclear distinction between the terms "severe" and "serious" AE (Candy et al., 2018, Harvey et al., 2014). Some studies used multiple metrics for symptoms of the gastrointestinal tract (GI), skin or airways. The symptoms reported as AE (number or % of participants experiencing symptoms) are presented here as sub-categories of AE, whereas other scales or metrics are presented under the sections 3.12.9 (GI), 3.12.10 (skin) and 3.12.11 (airways).

Reports of at least one or any AE ranged from 15% to 91% in the test groups and from 19.8% to 88% in the control groups. Reports of severe adverse events ranged from 0 to 14% in the test groups and from 2.9% to 24% in the control groups. Of the ten studies, three reported higher prevalence of at least one AE or mild AEs in the test group than in the control group (Burks et al., 2015; Van der Aa et al., 2010; Wang et al., 2021). The difference in prevalence varied from 10% to 19% but was not reported as statistically significant. These three studies comprised both healthy and allergic infants, including infants with atopic dermatitis who received *B. breve* M-16V in concentrations of $4.1x10^8$-$1.47x10^9$ cfu per 100 mL formula for 12 to 16 weeks. Four other studies reported higher prevalence of AE in the control group than in the test group (Abrahamse-Berkeveld et al.,2016; Chatchatee et al., 2022; Fox et al., 2019; Harvey et al., 2014, study 1). Here, the difference in prevalence varied from 9% to 25% but was not reported as statistically different. These four studies comprised both healthy and allergic infants who received *B. breve* M-16V in concentrations of $1.3x10^9$-$1.47x10^9$ cfu per 100 mL formula for eight weeks to 12 months. The last three studies had no report of AE or reported no differences in AE between study groups and no SAEs (Harvey et al., 2014, study 2; Chua et al., 2017; Phavichitr et al., 2021).

Six studies did comment on the relation of AE to the study products, test and control formula. Four studies considered that the AE/SAEs were not related or unlikely to be related to the study formulas. One study reported that subcategories of AEs related to the study product was comparable between groups, 23.5% in test and 24.3% in control group (Wang et al., 2021), and one study reported on subcategory of AE in two subjects that were related to the prebiotic and control formula, respectively (Chua et al., 2017).

Dropout due to adverse events ranged from two to 12 subjects in test groups and from one to 15 subjects in control groups. Three studies did not provide such information (Chatchatee et al.,2022, Chua et al., 2017, van der Aa et al., 2010).

Table 3.12.6-1 Results on adverse events from studies on infant formula.

Author, year	Adverse events overall test/control	Result sub-categories test/control	Treatment relation	Definition AE	Dropouts AE test/control	VKM note
Abrahamse-Berkeveld et al., 2016	At least one (serious) AE: 15.0%/19.8% (Fisher's exact test p=0.372). SAE: see AE		Nothing reported	Serious AE: any untoward medical occurrence resulting in death, is life-threatening (at the time of the event), requires hospitalisation or prolongation of existing hospitalisation or results in persistent or significantly disability or incapacity	Dropouts atopic dermatitis 2/0, constipation 4/5, diarrhoea 2/1, vomiting 2/9. No sig. difference between groups	No. of SAE not possible to separate from total AE. Study in healthy infants, development of dermatitis or diarrhoea (≥3 liquid stools per d for >1 week) were *a priori* defined protocol violations
Burks et al., 2015	At least one AE: 43/38 or 80%/68%. SAE: 2/4 or 3.7%/7.1%.	Mild AE: 24/14 or 44%/25% (p<0.05). Gastrointestinal system disorders Diarrhoea: 12/2 or 22%/4% (p=0.004) Skin and appendages disorders: 20/14 or 37%/25% (p ≥ 0.05) Respiratory system disorders: 29/23 or 54%/41% (p ≥ 0.05)) (Fisher's exact test, all p-values)	All SAEs and diarrhoea were assessed by the investigators as being unrelated to the study formula	Serious AE not defined	6/3	
Candy et al., 2018 (ASSIGN 0-8 wks)	Any AE: 20/23 or 57.1%/65.7%. (Fisher's exact test p=0.624). SAE: not reported. Severe AE: 1/1 or 2.9%/2.9%	Gastrointestinal disorders 11/13 or 31.4%/37.1% (Fisher's exact test p=0.802	Nothing reported	Reported severe adverse events were feeding disorder (test) and bronchiolitis and feeding disorder (control)	2/0	Unclear distinction between “severe” adverse event, and “serious” adverse events (SAE)

Author, year	Adverse events overall test/control	Result sub-categories test/control	Treatment relation	Definition AE	Dropouts AE test/control	VKM note
Fox et al., 2019 (ASSIGN 0-26 wks)	Any AE: 25/28 or 71%/80%. (Fisher's exact test p=0.578). SAE: 3/2 or 8.6%/5.7%.	Gastrointestinal disorders: 15/17 or 42.9%/48.6%, Fisher's exact test p=0.811	All SAE were considered not related, or unlikely to be related, to the study product.	Types of reported (serious) AE were gastroesophageal reflux disease, laryngitis viral, and bronchiolitis that required hospitalization (n = 2), and an anaphylactic reaction to pineapple	3/0	
Chatchatee, et al., 2022	Any AE: 70/75 (88%/84%) (p=0.549). Any SAE: 11/21 or 14%/24% (Miettinen-Nurminen p=0.104)		Nothing reported	Serious AE categorized as hospitalizations, mainly due to infections (GI/diarrhoea and respiratory infections)	No information	
Chua et al., 2017	AE: no difference between groups (Chi-square p=0.62)	Post-hoc analysis showed a lower percentage of subjects with AEs-related skin disorders in the synbiotic compared to the control group (20% vs 42%, p=0.017)	All reported AEs not related to the study product except for one subject in the prebiotic and the control groups (irritability and constipation of mild severity, respectively		No information	Data not shown.
Harvey et al., 2014 (study 1)	At least one AE: 37/49 or 63%/88% (no p-value). SAE 4/4 or 6.8%/7.1%	Reported as definitely, probably, or possibly related to formula: Constipation 2/13 or 3%/23%, diarrhoea 2/1 or 3%/2%, gastroenteritis 2/1 or 3%/2%, spitting up 2/3 or 3%/5%, vomiting 4/4 or 7%/7%, eczema 0/2 or 0%/4%	Most of AEs categorized as mild and unrelated to the formulations		12/10	Unclear distinction between “serious” adverse events (SAE) in text and “severe” adverse event (SAE) in tables. AE sub-categories stratified by “related” or “unrelated” to formula. Only “related” presented by VKM
Harvey et al., 2014 (study 2)	Any AE: not reported. SAE: 0		Nothing reported			
Phavichitr et al., 2021	AE: 1/1/0 (3 groups), SAE: 0		Nothing reported		1/1/0	

Author, year	Adverse events overall test/control	Result sub-categories test/control	Treatment relation	Definition AE	Dropouts AE test/control	VKM note
van der Aa et al., 2010	AE: 91.1%/84.1% (Chi square p=0.35). SAE 2/0 or 4.4%/0%		None of the reported AEs considered to be treatment-related	AE defined as any symptom or disease episode that occurred during the study. Serious AE: hospitalization because of respiratory syncytial virus bronchiolitis and because of severe cow's milk allergy in the synbiotic group	No information	
Wang et al., 2021	AE: 52.9%/43.0% (p= 0.15). At least one SAE: 5/3 or 4.9%/2.8% (p=0.43)	Skin and subcutaneous tissue disorders 22/20 or 21.6%/18.7%, with eczema being the most reported: 18/17 or 17.6%/15.9%. GI disorders also commonly reported: 16/13 or 15.7%/12.1%	AEs related to the study product was comparable between groups: 24/26 or 23.5%/24.3%, mostly skin and subcutaneous tissue disorders followed by GI disorders. None of the AEs reported as related to the study product were classified as severe	Serious AE: any untoward medical occurrence resulting in death, is life-threatening (at the time of the event), requires hospitalisation or prolongation of existing hospitalisation or results in persistent or significantly disability or incapacity	3/2	Interim analysis to review AEs conducted after 109 randomized infants completed the visit at 17 wk of age and was evaluated by an independent data monitoring committee. It was recommended that the study continue without modification

3.12.7 Results formula feeding – infant growth

Infant growth was assessed in ten publications and nine studies (all RCTs): (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Chatchatee et al., 2022; Chua et al., 2017; Fox et al., 2019; Harvey et al., 2014; Phavichitr et al., 2021; van der Aa et al., 2010; Wang et al., 2021). Harvey et al., 2014 only contributed results from Study 1. A total of 706 infants received *B. breve* M-16V in test formula and 648 received control formula (study descriptions in Table 3.12.4-1 and Table 3.12.4-2). All studies reported on infant growth indicators during the intervention period.

Three studies defined growth as a primary outcome (Burks et al., 2015, Harvey et al., 2014, Wang et al., 2021). Most studies measured growth at repeated time points and reported either Z-scores alone (Burks et al., 2015; Chatchatee et al., 2011) or in combination with absolute change in weight and length for age (Abrahamse-Berkeveld et al., 2016, Fox et al., 2019, Harvey et al., 2014; Wang et al., 2021). Four studies did not specify the growth indicators that were measured (Candy et al., 2018; Chua et al., 2017, Phavichitr et al., 2021, van der Aa et al., 2010). Fox et al., 2019 was a follow-up study of Candy et al., 2018 (ASSIGN study) and reported more detailed results and for the whole study period (up to week 26). Therefore, the results in Fox et al. are emphasized.

All studies that reported results in relation to a growth standard, used the standard from WHO 2006. No deviations from the standard were reported (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Chatchatee et al., 2022). Four studies reported anthropometric results as “no difference between groups” or “both groups grew well according to standards” (Chua et al., 2017 Fox et al., 2019, Phavichitr et al., 2021, van der Aa et al., 2010). Only one study reported a difference in growth between groups. Abrahamse-Berkeveld found significantly lower absolute length in the test group compared to controls in the ITT-population, a difference of 0.9 cm after 13 weeks. However, the difference in Z-scores was close to zero in the PP-population (Abrahamse-Berkeveld et al., 2016).

Table 3.12.7-1. Results on infant growth from studies on infant formula.

Author, year	Indicator measured	Result as reported	VKM note
Abrahamse-Berkeveld et al., 2016	Weight gain (d/day)	Mean (SD) g/day not statistically different. ITT population: test 27.5 (0.7) vs control 28.5 (0.7). PP-population: test 28.7 (6.4) vs control 29.8 (6.0), 13 wks. No p-values Growth in test and control groups considered equivalent when 90 % CI of the difference in means laid within the equivalence margins ± 0.5 SD, ± 3.1 g/d. ITT population: -1.4 (90 % CI: −3.0 to +0.3 g/d), PP population: -1.3 (90 % CI: −3.3 to +0.8 g/d), ANCOVA	
	Length gain (cm/week), and absolute length	Tendency of lower mean (SD) cm/week in test group. ITT population: test 0.77 (0.02) vs control 0.81 (0.02) (p=0.093). PP population: test 0.77 (0.13) vs control 0.82 (0.16) (p=0.11), 13 wks. ANCOVA tests. Lower absolute length (cm) in the test vs. control group at all wks (4, 8, 13). Week 13 mean (SD) test 62.5 (0.3) vs control 63.4 (0.3). ANCOVA p=0.022	
	Weight-for-age z-score	Close to zero in both groups. PP population: repeated measures mixed model p=0.102 (test) vs p=0.130 (control)	WHO 2006 growth standard
	Length-for-age z-score	Close to zero in both groups. PP population: repeated measures mixed model p= 0.868 (test) vs p=0.331 (control)	
Burks et al., 2015	Weight z-score	Difference (90% CI) in weight z-scores (test–control) was 0.147 (-0.10, 0.39), p = 0.32 at week 16 Equivalence margin for z-score = 0.429, based on smallest meaningful difference in infant growth increments of 3 g/d recommended by American Academy of Paediatrics	WHO 2006 growth standard
	Length z-score	Difference (90% CI) in length z-score (test–control) was -0.299 (-0.69, 0.09), p = 0.21 at week 16	As above
	Weight-for-age z-score	Z-score (mean ± SEM) not significantly different between test and control groups, no p-value	As above
	Length-for-age z-score	Z-score (mean ± SEM) not significantly different between test and control groups, no p-value	As above
Candy et al., 2018, see Fox et al., 2019	Standard anthropometric measurements (not specified)	Growth within expected ranges for age, and median z-scores within 1 SD of the mean for both test and control groups	Data not shown. Unknown growth standard
Fox et al., 2019 (ASSIGN)	Weight, weight gain, length, length gain	Not statistically significantly different between test and control groups at any time points (to wk 26). All growth parameters within expected ranges for age	Unknown growth standard
	Weight-for-age z-score	As above	As above
	Length-for-age z-score	As above	As above
	Weight-for-length z-score	As above	As above

Author, year	Indicator measured	Result as reported	VKM note
Chatchatee et al., 2022	Weight-for-age z-score	Mean z-scores within range + 0.5 to - 0.5 over 12 months, no difference (mean ± SEM) between test and control	Data in figures. WHO 2006 growth
	Length-for-age z-score	As above	As above
	Weight-for-length z-score	As above	As above
Chua et al., 2017	Anthropometry (not specified)	All groups (test synbiotic, test prebiotic, control) showed a comparable safety profile that included growth	Data not shown
Harvey et al., 2014 (study 1)	Weight gain (test, control) over time	No statistically significant difference in weight gain between groups. The ratios (90% CI) for test/control were 0.98 (0.95, 1.01) (ITT) and 1.00 (0.97, 1.04) (PP)	Data in figure
	Ratio length 2 groups (test, control) over time	No statistically significant difference in achieved length between groups. The ratios (90% CI) for test/control were 0.99 (0.98, 1.00) (ITT) and 0.99 (0.98, 1.01) (PP)	
Phavichitr et al., 2021	Anthropometry (not specified)	Infants grew well according to WHO child growth standards	Data not shown.
van der Aa et al., 2010	Growth/weight	Linear and ponderal growth did not differ between the two groups	Data not shown
Wang et al., 2021	Weight gain	Difference in means (SE) between test and control group was -0.36 (0.93) g/d (90% CI: -1.90 to 1.18), PP population. Robust to sensitivity analyses	
	Length gain	Difference in means (SE) between test and control group was 0.01 (0.02) mm/d (90% CI, - 0.05 to 0.03), PP population. Robust to sensitivity analyses	
	Weight-for-age z-score	Mean z-score within or close to ± 0.5 SD bandwidth for test and control (to wk 17)	Data in figures, WHO 2006 growth standard
	Length-for-age z-score	As above	As above
	Body mass index-for-age z-score	As above	As above
	Mid upper arm circumference -for-age z-score	Measured at 13 and 17 wk only. Mean z-score between +0.5 and +1 SD bandwidth at 13 wk and close to +1 SD at 17 wk	As above

3.12.8 Results formula feeding – gastrointestinal (GI) symptoms

There were nine publications on GI-symptoms (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Chua et al., 2017; Fox et al., 2019; Harvey et al., 2014; Sorensen et al., 2021a; van der Aa et al., 2010; Wang et al., 2021) from eight studies. All had an RCT design, except Sorensen et al., 2021a (retrospective observational). A total of 537 infants received *B. breve* M-16V in test formula and 551 received control formula.

VKM summarized GI-symptoms considerd to have potentially high clinical relevance if severe: diarrhoea, constipation, vomit, and regurgitation. (For other GI-symptoms not summarized, see section 3.12.13). In most studies, the GI symptoms were reported by parents/caregivers, usually prospectively in diaries, and stated to be evaluted by clinicians in some of the studies. Of note, Abrahamse-Berkeveld et al., 2016 included healthy children and diarrhoea (≥3 liquid stools per d for >1 week) was considered a protocol violation and reason for drop-out during the study (2 reports in test group vs 1 in controls). Sorensen et al., 2021a extracted case records from a clinical database and presented GI symptoms overall but was included.

One study reported GI-symptoms as a measure of tolerance and concluded that the study formula was well tolerated. Six studies reported that there were no differences in prevalence of GI-symptoms between study groups. The observational study reported that significantly fewer infants reported GI-symptoms in the synbiotic group than in the control group (Sorensen et al., 2021a).

Table 3.12.8-1. Results on gastrointestinal (GI) symptoms from studies on infant formula.

Author, year	GI symptom	Classifications used	Result as reported	VKM note
Abrahamse-Berkeveld et al., 2016	Diarrhoea severity	Severity scores: 0 = absent; 1 = mild; 2 = moderate; 3 = severe.	Mean values not significantly different from that for the control group (P≥ 0.05) at any of 3 timepoints (0-4, 4-8, 8-13 weeks)	Diarrhoea (≥3 liquid stools per d for >1 week) considered protocol violation (test/control 2/1)
	Constipation severity	As above	As above	
	Vomiting severity, regurgitation severity.	As above	As above	
Burks et al., 2015	Allergic symptoms, including spitting up and vomiting	Allergic symptoms categorized as 'none,' 'slight,' 'moderate,' 'severe' and 'very severe'	Allergic symptoms, including spitting up and vomiting, not different between groups.	Data not shown
Candy et al., 2018; Fox et al., 2019	Vomiting (wks 1 ,4, 8, 12, 26).	4-point rating scale: 1: none, 2: one to two times/ day, 3: three to four days/day, and 4: more than four times/day	Mean (95% confidence limits) scores reduced in both groups towards the lowest possible score (1 = none), not statistically significantly different between groups (Fox et al.)	Exploratory analyses. Data in figures
	Spitting up (wks 1 ,4, 8, 12, 26).	4-point rating scale: none, 2: after some feeds, 3: after all feeds, and 4: between and after feeds	As above	
Chua et al., 2017	Unspecified gastrointestinal tolerance	Not reported	All formulas were well tolerated	Indicators not specified. Data not shown
Harvey et al., 2014 - study 1	Spitting up, vomiting (wks 2 ,4, 8, 12, 16)	4-point rating scale: 1 = none, 2 = slight, 3 = moderate, 4 = severe	No associations between study product and the most frequent category recorded for spitting up (category 2), vomiting (category 1)	
Sorensen et al., 2021a	Case records on GI symptoms from the UK Health Improvement Network (THIN) database.	Examples of symptoms classified as GI included GI illness, diarrhoea, constipation, flatulence, vomiting, reflux, bloody stools, mucus in stools and colic.	Significantly fewer infants experienced GI symptoms with synbiotic than control formula, expressed as proportion (23% vs 46%, Chi-square p=0.006) or rate (0.43 vs 0.72 per person year, Poisson test p=0.013)	Not possible to separate different symptoms
van der Aa et al., 2010	Diarrhoea (wks 0, 4, 8, 12)	An episode of three or more watery stools in 24 h was considered as diarrhoea.	Diarrhoea occurred equally in both groups, test/control n or %: 17/12 or 37.8%/27.3% (Chi-square p=0.37)	
	Constipation (wks 0, 4, 8, 12)	Not defined	Less constipation in test group, test/control n or %: 0/6 or 0/14%, Fisher's exact test p=0.01	

Author, year	GI symptom	Classifications used	Result as reported	VKM note
van der Aa et al., 2010	Gastroenteritis (wks 0, 4, 8, 12)	Any episode of vomiting and diarrhoea with or without fever was considered as gastroenteritis	Gastroenteritis occurred equally in both groups, test/control n or %: 6/2 or 13.3%/4.5%, Fisher's exact test p=0.27	
Wang et al., 2021	Vomit and regurgitation, (age < 2, 4, 8, 13, 17 wks)	Return of the milk into the mouth 1) without force= regurgitation, 2) with force=vomiting: Analysed as regurgitation or vomiting at least once on 1 day, and the occurrence of frequent regurgitation or vomiting (2 to 3 days with three or more regurgitation or vomiting episodes)	No statistically significant differences in the occurrence of regurgitation or vomiting between the formula groups at any time point	

3.12.9 Results formula feeding – skin symptoms

There were nine publications (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Chatchatee et al., 2022; Fox et al., 2019; Hulshof et al., 2018; Sorensen et al., 2021a; Taniuchi et al., 2005; van der Aa et al., 2010) that reported on allergic or atopic skin symptoms from eight studies. All had an RCT design, except Sorensen et al., 2021a (observational). A total of 420 infants received *B. breve* M-16V in test formula and 444 received control formula.

Abrahamse-Berkeveld et al., 2016 was conducted in healthy infants and development of atopic dermatitis was considered a protocol violation (2 reports in test group vs 0 in controls, but not statistically different). The other studies included infants with confirmed (Ig-E mediated) or suspected (non-Ig-E mediated) cow's milk allergy, atopic dermatitis, or both. Due to some variations in the result presentations in Candy et al., 2018 and Fox et al., 2019 (ASSIGN study), both are shown.

Six of eight studies included clinician-reported skin symptoms via the SCORing Atopic Dermatitis (SCORAD) rating scale or a modified version of SCORAD. Two of six studies also used parental report of skin symptoms in addition to SCORAD (Burks et al., 2015; Fox et al., 2019). In these studies, VKM only summarized SCORAD. The last two of eight studies were based on either case records extracted from the UK Health Improvement Network (THIN) database where examples of skin symptoms were eczema and urticaria (Sorensen et al., 2021a), or a cutaneous symptom score (Taniuchi et al., 2005). Two studies also reported the percentage of subjects with skin symptoms as a sub-category of adverse events (Chua et al., 2017; Burks et al., 2015). These results are reported in the table for adverse events (Table 3.12.6-1).

Seven studies reported no differences in skin symptoms according to SCORAD between the study groups. Also, several studies reported that skin symptoms decreased over time in both test and control group. In the observational study significantly fewer infants experienced skin symptoms with synbiotic than control formula, expressed as a proportion (11% vs 26%, Chi-square p=0.033) or rate (0.17 vs 0.32 per person year (Sorensen et al., 2021a).

Table 3.12.9-1. Results on skin symptoms from studies on infant formula.

Author, year	Skin symptom	Result as reported	VKM note
Abrahamse-Berkeveld et al., 2016	SCORing Atopic Dermatitis (SCORAD) rating scale	During and at end of study, no statistically significant differences between the two groups (no p-value)	Data not shown. SCORAD reported collectively with other atopic symptoms. Development of atopic dermatitis considered protocol violation (2 in test vs 0 in controls, not statistically significant)
Burks et al., 2015	SCORAD	SCORAD decreased in both groups. Neither decrease from baseline, nor number of subjects with score of 0, differed significantly between the groups at weeks 4 or 16 (no p-value)	Data in figure
Candy et al., 2018 (ASSIGN)	SCORAD	SCORAD (mean ± SD) decreased in both groups from 12.83 ± 18.84 to 9.63 ±12.45 in the test group and from 14.43 ± 19.74 to 7.06 ±10.01 in the control group (between wks 0 and 8). No statistically significant changes (no p-value)	Results weeks 0-8, reported for weeks 0-26 in Fox et al., 2019
Fox et al., 2019 (ASSIGN)	SCORAD	SCORAD decreased in both groups. Median scores decreased by 6.0 (Q1–Q3: − 13.0, 0.0) and 7.0 (Q1–Q3: − 13.0, 0.0) in the test and control groups, respectively (between week 0 and 26)	
Chatchatee et al., 2022	SCORAD	Clinical symptoms decreased over time with test and control formula. No differences between the groups at 6 and 12 months	Data not shown. SCORAD reported collectively with other clinical symptoms
Hulshof et al., 2018	Objective SCORAD (oSCORAD), excluding subjective symptoms (e.g., pruritus, sleep disturbances)	Average oSCORAD decreased in those who completed the study. Median change in both groups of −8, resulted in a median (Q1–Q3) post-intervention oSCORAD of 13 (12–17) in the test group and 17 (12–21) in control group	
van der Aa al., 2010	SCORAD	SCORAD decreased in both groups: 12.7 points in the test group ($p<0.001$) and 14.5 points in the placebo group ($p<0.001$), paired-samples t-test. However, no statistically significant difference in SCORAD change between the two groups at any of the time-points	
Sorensen et al., 2021a	Case records of skin symptoms from the UK Health Improvement Network (THIN) database (e.g., eczema and urticaria)	Significantly fewer infants experienced skin symptoms with synbiotic than control formula, expressed as proportion (11% vs 26%, Chi-square $p=0.033$) or rate (0.17 vs 0.32 per person year, Poisson test for rates $p=0.066$)	

Author, year	Skin symptom	Result as reported	VKM note
Taniuchi et al., 2005	Cutaneous symptom score. Modified scoring system for atopic dermatitis (erythema, lichenification, and cracking on face, trunk, arms, and legs, itching and sleep disturbance)	In the bifido-group, the cutaneous symptom scores at 1, 2, and 3 months decreased significantly (p=0.03, 0.01, 0.04, respectively) compared to the pre-administration score. In the control group, the cutaneous symptom scores decreased significantly only at 1 and 3 months (p=0.04, not reported, 0.04, respectively). P=Wilcoxon matched pairs signed-rank test	Data in figure

3.12.10 Results formula feeding – airway symptoms

There were six publications on airway symptoms (Abrahamse-Berkeveld et al., 2016; Burks et al., 2015; Candy et al., 2018; Fox et al., 2019; Sorensen et al., 2021; van der Aa et al., 2011) from five studies. All had an RCT design, except Sorensen et al., 2021a (observational). A total of 314 infants received *B. breve* M-16V in test formula and 333 received control formula.

Abrahamse-Berkeveld, M. et al. 2016 was the only study carried out in healthy children without cow's milk allergy or atopic dermatitis.

As previously reported, Candy et al., 2018 and Fox et al., 2019 both reported on the ASSIGN study and Fox et al., 2019 reported on a study period of 26 weeks, including results to eight weeks in Candy et al., 2018.

Airway symptoms were defined as atopic symptoms in airways, allergic respiratory symptoms, respiratory symptoms, or asthma-like symptoms such as noisy breathing or wheezing without cold. The symptoms were either physician monitored at study visits or parent reported. Four of the five studies found that there were no differences in airways symptoms between the study groups. Two studies reported that the airway symptoms were low or decreased over time. One study reported significantly higher prevalence of wheezing and noisy breathing in control than in test group (van der Aa et al., 2011).

Table 3.12.10-1. Results on airway symptoms from studies on infant formula.

Author, year	Airway symptom	Classifications used	Result as reported	VKM note
Abrahamse-Berkeveld et al., 2016	Atopic symptoms of airways	No classification. Physician monitored at hospital visits	During and at the end of the study, no differences were statistically significantly confirmed in atopic symptoms between the two groups (no p-value)	Data not shown. Atopic symptoms reported collectively
Burks et al., 2015	Allergic symptoms, respiratory	Parent-reported allergic symptoms categorized as 'none,' 'slight,' 'moderate,' 'severe' and 'very severe'	Allergic symptoms were not different between groups	Data not shown
Candy et al. 2018; Fox et al., 2019	Respiratory symptoms (blocked nose, coughing, and wheezing) (wk 0, 1 ,4, 8, 12, 26)	Parent-reported rating scales. Blocked nose and wheezing rated as 1: none, 2: mild, 3: moderate, and 4: severe, and coughing was rated as 1: none, 2: one to two times/day, 3: three to five times/day, and 4: more than five times/day	No statistically significant differences were observed at week 8 (Candy et al.) Respiratory related symptoms reduced over time (coughing, blocked nose), or remained similar to level at study entry (wheezing, close to 1 at study entry) (Fox et al.)	Exploratory analyses. Data in figures
Sorensen et al., 2021a	Case records from the UK Health Improvement Network (THIN) database.	Symptoms classified as respiratory included asthma and rhinitis.	Recorded respiratory symptoms were low across the entire cohort, with no significant differences between groups: (0% vs 2.7%, Chi-square p=0.5) or rate (0 vs 0.02 per person year, Poisson test p=0.5)	
van der Aa et al., 2011	Asthma-like symptoms: Frequent wheezing (≥ 3 episodes after intervention period)	Physician assessed. Parents asked about respiratory symptoms (cough, shortness of breath, noisy/rattly breathing, wheezing) and medication use of their child, using a validated questionnaire	Test/control 5/13 or 13.9%/34. 2%, Chi-square p=0.04. Absolute risk difference (95% CI):= -20.3 (-39.2, -1.5)	
	Asthma-like symptoms: Wheezing apart from colds:	As above	Test/control 1/7, or 2.8%/17.9%, Chi-square p=0.056. Absolute risk difference (95% CI)= -15.2 (-28.4, -2.0)	
	Wheezing and/or noisy breathing apart from colds:	As above	Test/control 1/12, or 2.8%/30.8%, Chi-square p=0.001. Absolute risk difference (95% CI)= -28.0 (-43.4, -12.5)	

3.12.11 Confidence ratings formula feeding – adverse events and growth

VKM generated evidence profiles and confidence ratings for the body of evidence found on the association of infant formula intake with two health outcomes: adverse events (section 3.12.6) and growth (section 3.12.7). These outcome groups were considered to have the highest clinical relevance among the reported outcomes. In all studies, adverse events and growth were assessed during the intervention period (duration six to 17 weeks except one study lasting 12 months) and therefore considered as evidence of short-term health effects according to VKM's definition.

The body of evidence was similar for both outcomes and consisted of the same ten publications contributing results from ten (adverse events) or nine (growth) studies, all with RCT design. The individual study characteristics have been described in section 3.12.4, risk of bias assessment in section 3.12.5, and study results in section 3.12.6 (adverse events) and section 3.12.7 (growth) which are all used when assessing confidence. Based on the study design (only RCTs) the initial confidence rating was "high" for both outcomes (Table 3.12.11-1). VKM's overall confidence in the body of evidence was downgraded from "high" to "moderate" for both outcomes meaning that "the true effect may be reflected in the apparent relationship" (section 2.2.8).

Summary of downgrading elements: the initial confidence rating "high" was downgraded to "moderate" for the outcome adverse events due to unexplained inconsistency, imprecision and suspected publication bias. Because it was difficult to distinguish between inconsistency and imprecision, the outcome adverse events was only downgraded once for one of the domains, in accordance with the OHAT handbook (OHAT, 2019). The outcome infant growth was downgraded to "moderate" due to suspected publication bias. VKM evaluated other factors (risk of bias, indirectness) as "not serious". The evaluation of the different elements is explained in more detail below.

Risk of bias: Most of the information was from studies in Tier 1 for both outcomes (section 3.12.5) indicating that risk of bias is not serious (i.e., unlikely to seriously alter the results). Key questions in the risk of bias evaluation were blinding, exposure characterization, and outcome assessment.

Inconsistency: This refers to large variability in the direction or magnitude of individual study effect estimates for comparable measures of association that cannot be explained. For adverse events, the percentage of the test and control groups experiencing AE varied widely across studies, possibly due to differences in definitions of AE and/or assessment methods, study populations (healthy or allergic/atopic infants) and study duration. However, the result emphasized by VKM were differences between groups (test or control) within the same study. These differences varied less, but in both directions (% AE appeared higher in controls in some studies, higher in the test group in some studies, or similar) also among studies in Tier 1 for risk of bias. Reported statistical tests were not significantly different, except in some subgroups of AE. Because it was difficult to determine to what extent the variability reflects differences in AE or imprecision, VKM evaluated both the domains inconsistency and imprecision (described below) as "serious" but only downgraded once.

Imprecision: This refers to the ability of studies to distinguish treatment from controls, and the confidence interval (CI) around effect estimates. OHAT uses 95% confidence intervals as the primary method to assess imprecision. OHAT also considers whether the studies are adequately powered when assessing precision, which is especially important when interpreting findings that do not provide support for an association (OHAT, 2019). The percentage of the test and control groups experiencing AE were generally presented without CIs or standard errors, and none of the studies provided power calculations for AE. Numbers were generally very small for serious adverse events and/or drop-out due to AE and differences between groups were difficult to evaluate.

Indirectness: This refers lack of or low applicability when addressing the objectives of the evaluation. Because all studies assessed formula intake in infants, and health outcomes (AE and growth) were measured directly, indirectness was not considered an issue.

Publication bias: According to the OHAT guideline, "publication bias should be suspected when studies are uniformly small, particularly when sponsored by industries, non-government organizations (NGOs), or authors with conflicts of interest. When possible, OHAT will evaluate findings by funding source or by whether the author(s) reported a conflict of interest." VKM concluded that publication bias is "strongly suspected" on the basis that all studies had authors affiliated with the manufacturers of *B. breve* M-16 V or infant formula, and few studies had safety as a main study objective or primary outcome. Thus, it was not possible to evaluate findings by funding source.

Summary of upgrading elements: Because the body of evidence did not clearly support an association between intake of formula and health risk (adverse effects or growth disturbances), the upgrading factors (large magnitude and dose-response) were not considered relevant.

Nature of health effects: VKM did not find consistent differences in the occurrence or type of side effects or growth of infants in the test and control groups in studies on infant formula. Thus, VKM considered the "nature" of the effect (section 2.2.8) of formula containing *B. Breve* M-16V to be "no health effect". However, when the confidence in the body of evidence is "moderate", the evidence is not considered sufficient to determine with certainty that *B. Breve* M-16V is not associated with these health outcomes.

Table 3.12.11-1. Evidence profile for infant formula studies on two health outcomes: adverse events and growth.

	Elements triggering downgrading					Elements triggering upgrading				
Body of evidence	Risk of Bias	Unexplained inconsistency	Indirectness	Imprecision	Publication bias	Magnitude	Dose-response	Residual confounding	Consistency across species/model	Final rating
Response categories	Serious or not serious	Serious or not serious	Serious or not serious	Serious or not serious	Undetected or strongly suspected	Large or not large	Yes or no	Yes or no	Yes or no	High, moderate, or low
Health outcome: adverse events										
10 RCTs in infants, initial rating=HIGH	Not serious	Serious	Not serious	Serious	Strongly suspected	Not large	No	No	Not evaluated	MODERATE
Health outcome: growth										
9 RCTs in infants, initial rating=HIGH	Not serious	Not serious	Not serious	Not serious	Strongly suspected	Not large	No	No	Not evaluated	MODERATE

3.12.12 Results enteral feeding – review and primary studies

Two reviews were used to assess health risk related to *B. breve* M-16V in enteral feeding of preterm infants. The first review by Athalye-Jape et al., 2018 (Table 3.12.12-1.) included a total of 2978 preterm (<37 weeks) low birth weight infants (<2500 g) from five RCTs and four non-RCTs. Enteral administration of any dose of *B. breve* M-16V started within the first 10 days of life, continued for at least seven days, and was compared with placebo or control. The dose of *B. breve* M-16V ranged from 0.5 to 3 × 10^9 cfu per day, and the supplementation was given from start of enteral feeding till at least 37 completed weeks of gestation or discharge.

For the outcomes stage ≥2 NEC, all-cause mortality, late-onset sepsis and postnatal age at full feed, the authors concluded with no beneficial effect of *B. breve* M16-V based on RCT studies, but significant reduction in non-RCTs. A pooled estimate based on the relative risk (RR) or odds ratios (OR) was provided, when possible, based on two or three studies. None of the studies in the review reported any unintended or worsening effect of the outcomes included. The quality of evidence was graded for outcomes of critical importance: stage ≥2 NEC, all-cause mortality, and late-onset sepsis. The grade was "very low" for all three outcomes (Athalye-Jape et al., 2018). The author stated that for the outcome of stage 2 NEC, the overall quality of evidence from RCTs was downgraded to very low, in view of the limited number of RCTs (1 study), the very small sample size from RCTs, the wide confidence intervals around the effect size estimates, and the fact that it was a secondary outcome of interest in that study. The quality of evidence from non-RCTs was deemed to be very low due to the presence of the small number of studies, the confounding factors, and the possibility of changes in clinical practice over time. Eight studies, both RCTs and non RCTs, reported no cases of sepsis due to probiotics. The review authors emphasize that current evidence is limited to reflect the true risk at the population level when using this strain for routine probiotic supplementation.

VKM identified four primary studies on enteral feeding published after the review by Athalye-Jape et al. from 2018: Agrawal et al., 2020; Athalye-Jape et al., 2020; Inage et al., 2022; Priyadarshi et al., 2021). A total of 472 preterm infants were given 2.5 × 10^9 and 3.0 × 10^9 cfu per day by the enteral route. For the outcomes stage ≥2 NEC, late-onset sepsis, weight and length Z-scores, there were no new findings as compared to the review. Priyadarshi et al., 2021 reported no findings of probiotic induced sepsis and thus supports the empty review of Kulkarni et al., 2022. One of the studies was a follow-up of preterm infants for neuropsychological development 3-5 years after the intervention with *B. breve* M-16V, with no negative effects (Agrawal et al., 2020). Inage et al., 2022 studied risk factors for hyperglycemia in extremely low birth weight infants (<1000 g) and found *B. breve* M-16V to increase risk with an adjusted hazard ratio (HR) of 2.95 with a wide 95% confidence interval (95% CI: 1.10, 7.87; p=0.031). The authors pointed to dextrin used as an excipient in *B. breve* M-16V as a likely explanation, not the bacteria per se.

Table 3.12.12-1. Summary of results on enteral nutrition and complications in premature/low-birth-weight infants (Athalye-Jape et al., 2018).

Outcome	RCTs (total N)	Non-RCTS (total N)	Meta-analysis: (RCTs and non-RCTs): Random effects models	Author conclusions	Author grading of evidence[1] (RCTs and non-RCTs)
Stage ≥ 2 necrotizing enterocolitis (NEC)	1 study (159)	1 study (1755)	Not possible	RCTs did not show beneficial effects, non-RCTs showed sig. reduction	GRADE Very low
All-cause mortality	2 studies (361)	2 studies (2319)	Not possible for RCTs (null events in one study). Non-RCT: OR=0.61 (95% CI, 0.44, 0.84); p=0.002; I^2 = 0%	RCTs did not show beneficial effect, non-RCTs showed significant reduction	GRADE Very low
Late-onset sepsis (LOS)	3 studies (368)	3 studies (2452)	RR=0.71 (95% CI, 0.31, 1.62); p=0.42; I^2 = 55% OR=0.53 (95% CI, 0.35, 0.81); p=0.003; I^2 = 57%.	RCTs did not show beneficial effect, non-RCTs showed significant reduction	GRADE Very low
Postnatal age at full feeds	2 studies (361)	2 studies (1888)	RR= −2.05 (95% CI, −9.29, 5.18); p=0.58; I^2 = 88% OR=−2.42 (95% CI, −2.55, −2.3); P<0.00001; I^2 = not estimable.	RCTs did not show beneficial effect, non-RCTs showed significant reduction	Not available
Probiotic sepsis	5 studies (482)	3 studies (2452)	Not possible, no events	No adverse effects (including *B. breve* M-16V sepsis) in any included studies.	Not available
Stage ≥ 2 NEC related mortality	0 studies	1 study (133)	Not possible, 1 study	NEC-related mortality reduced in the *B. breve* M-16V group (5.6% vs 0%)	Not available
Weight gain	1 study (208)	0 studies	Not possible, 1 study	Weight gain was significantly better in probiotic vs control	Not available
Duration of hospitalization	3 studies (380)	1 study (1755)	RCTs: RR = –0.25 (95% CI, –0.66, 0.15); p=0.22; I^2 = 0%	Hospital stay reduced by 0.25 days in probiotic vs control group	Not available

[1]Grade according to Grading of Recommendations, Assessment, Development, and Evaluation (GRADE)

The second review by Kulkarni et al., 2022 investigated sepsis due to probiotics in preterm neonates. Probiotic sepsis was defined as positive blood/CSF culture isolating administered probiotic strain with symptoms suggestive of infection. The review was considered empty as it did not find any studies or reports of sepsis due to *B. breve* M-16V.

Table 3.12.12-2 presents four primary studies on enteral feeding (a total of 472 infants exposed to *B. breve* M-16V) published after the review by Athalye-Jape et al., 2018. Two observational studies (Athalye-Jape et al., 2020; Priyadarshi et al., 2021) presented secondary outcomes similar to those in the review (time to reach full enteral feeds, stage ≥ 2 NEC, late-onset sepsis, and weight- and length Z-score at discharge, or probiotic sepsis) and two studies presented different outcomes: one observational study on hyperglycemia (Inage et al., 2022) and one RCT on neuropsychological development at age 3 to 5 years (Agrawal et al., 2020). In these studies, *B. breve* M-16V was administered via the enteral route in doses of 2.5×10^9 and 3.0×10^9 cfu per day, or there was not specific dose (Inage et al., 2022). The study subjects comprised 67 children at age 3 to 5 years who had participated as preterm infants (< 33 weeks) in one study, 154 extremely preterm infants (<28 weeks) and 55 extremely low birth weight infants (body weight <1000 g) 196 unspecified preterm infants in one study. The study of extremely low birth weight infants found that consumption of *B. breve* M-16V was related to hyperglycemia. Otherwise, there were no adverse effects or unintended consequences on stage ≥ 2 NEC, growth indicators, sepsis or neuropsychological development reported in the studies of *B. breve* M-16V administered by enteral feeding.

Table 3.12.12-2. Results from four primary studies on enteral feeding published after 2018.

Author, year	Outcome	Result as reported	VKM note
Athalye-Jape et al., 2020	Time to reach full enteral feeds (150 mL/kg/d) in days	Median (IQR) single strain 10 (8-15) vs control 14 (12-20) days, Kruskal-Wallis p=0.022 for three groups	Result not presented for the third group (three-strain probiotic arm of the SiMPro RCT). Controls from different trial (PANTS)
	Stage ≥ 2 NEC	N (%) of 1 (1.3%) in single strain vs 0 in controls, unspecified exact method p=0.568 for three groups	
	Late-onset sepsis, LOS	N (%) of 18 (24.0%) in single strain vs 7 (24.1%) in controls, p=0.472 for three groups	
	Weight z-score at discharge	Mean (SD) −0.79 (0.90) in single strain vs. −0.64 (1.82) in controls, p=0.824 for three groups	
	Length z-score at discharge	Mean (SD) -1.27 (1.76) in single strain vs. -1.07 (2.33) in controls, p=0.390 for three groups	
Priyadarshi et al., 2021	Probiotic-induced sepsis	Stated that there were no cases of probiotic-induced sepsis during the study	No comparator without *B. breve* M-16V, therefore sepsis was only outcome included by VKM. Two-strain probiotic prophylaxis was sequentially switched over after 2 years to single strain probiotic within a 4-year study period, in similar cohorts of preterm infants.
Inage et al., 2022	Hyperglycemia	*B. breve* M-16V use increased the risk of hyperglycemia, with an estimated hazard ratio of 2.95 (95% CI 1.10-7.87), p=0.031 (Cox proportional hazards model). Adjusted for gestational age, chorioamnionitis, postnatal intravenous glucocorticoids.	Authors point to dextrin used as an excipient in *B. breve* M-16V as possible explanation, not the bacteria per se

Author, year	Outcome	Result as reported	VKM note
Agrawal et al., 2020	Primary outcome: Mullen's Scale of Early Learning (MSEL)	Multivariable analysis of the MSEL composite score gave no evidence of a probiotic effect. Adjusted mean effect (95% CI) of probiotic: -2.7 (-8.5, 3.0), p=0.349. Complete case analysis (CCA), adjusted for gestational age, intrauterine growth restriction, Apgar<7 at 5 min and age at time of assessment.	Follow-up study. Information on intervention collected from Patole et al., 2014. Children followed-up more likely to be born at gestation <28 weeks than those who were not followed-up (49.3% vs 30.4%, p=0.016). Adjusted results emphasized. Results robust to multiple imputation of MSEL composite score and T scores due to missing data in the follow up group.
	(MSEL) -subscales	Fine motor, visual reception, receptive language, expressive language: no significant differences in T-score, age-standardized score, or % children below average	
	Secondary outcome: Developmental, Dimensional and Diagnostic Interview (3Di)	Statement of no significant difference in the 3-Di scores in probiotic vs control group children	Scores, including for subscales, reported by authors, but not by VKM.
	Tertiary outcome: Developmental NEuroPSYchological assessment–2nd Edition (NEPSYII)	Statement of no significant difference in the outcome measures between the probiotic vs control group children	Scores, including for subscales, reported by authors, but not by VKM.
	Tertiary outcome: Children's Communication Checklist–2nd edition (CCC-2)	The median speech scaled score in the CCC-2 was significantly higher in the probiotic group (medians 10 vs 6, p=0.023), and the overall median GCC score was non-statistically higher in the probiotic group (79 vs 63, p=0.056)	Scores, including for subscales, reported by authors, but not by VKM. Subscales: Speech, syntax, semantic, coherence, inappropriate initiation, stereotyped language, use of context, non-verbal communication, social relations, interests
	Tertiary outcome: Social Responsiveness Scale (SRS)	Statement of no significant difference in the outcome measures between the probiotic vs control group children	Scores reported by authors, but not by VKM
	Tertiary outcome: Vineland Adaptive Behavioural Scales–2nd edition (VABS-II)	Statement of no significant difference in the outcome measures between the probiotic vs control group children	Scores reported by authors, but not by VKM

3.12.13 Other outcomes reported for formula feeding

According to the study eligibility criteria (section 2.2.1) VKM excluded some health outcomes with unclear health risk (changes in faecal microbiota composition, stool characteristics, markers of immunomodulation and inflammation, C-reactive protein) and bacteremia without sepsis.

Examples of stool characteristics commonly reported in the included studies on formula feeding and excluded by VKM are stool frequency, colour, consistency, and pH, as well as faecal metabolites (short chain fatty acids and L-lactate). Markers of immunomodulation in faeces (secretory immunoglobulin A, eosinophil cationic protein, calprotectin, and alpha1antitrypsin) or serum (total serum IgE, specific IgE against food and inhalant allergens, and serum eosinophilic granulocytes) were reported in one study each and excluded according to protocol.

Four studies (Abrahamse-Berkeveld et al., 2016; Burks et al. 2015; Harvey et al. 2014; van der Aa et al., 2010) analysed blood samples for safety parameters. The biomarkers were unspecified markers of renal and liver function in two of the studies. Three studies reported no difference between the test and control groups, and one study reported statistically significant differences (haemoglobin, haematocrit, RBC and alkaline phosphatase) but within reference ranges. None of the studies reported measured values or the reference ranges used. The results were found to be poorly documented and have limited interpretability, and the summary was moved to Appendix 10.7.

The ability of probiotic strains to produce the D-lactic acid isomer should be considered in safety assessments (FAO/WHO, 2001; FDA, 2013). Two studies included D-lactate specifically (Abrahamse-Berkeveld et al., 2016; van der Aa et al., 2010) and reported a statistically significantly higher concentration in the test group than in the control group after the intervention. However, concentrations were highest at baseline in both studies. Also, VKM is not aware of any reference values for D-lactate in faeces and did not consider the result clinically interpretable.

Because it was not possible to pre-specify all outcomes in the literature, some reported outcomes were deemed by VKM to indicate unclear health risk and excluded a posteriori

VKM summarized results on infant growth and various symptoms from the gastrointestinal tract (GI), skin, and airways, but certain reported outcomes were excluded from the summaries:

- GI symptoms: colic, flatulence as well as crying frequency and sleeping behaviour.
- Skin symptoms: diaper dermatitis (nappy rash)
- Growth: head circumference

Medication use: encompassed different medications from topical skin symptom treatment to systemic antibiotics and difficult to summarize as one outcome.

Infections: VKM considered the methodology and reporting too inconsistent to summarize. VKM was uncertain of the specificity of these symptoms and to what extent they reflected infection.

All-cause symptoms and health-care usage: not summarized due to only one study.

4 Exposure calculations and scenarios

4.1 Overview of exposure calculations

VKM has estimated daily exposure to *B. breve* M16-V from intake of infant formula as full diet and as supplementary diet, as specified in the ToR. According to the current national guideline for infant feeding from the Norwegian directorate of Health (HDIR, 2017), complementary foods should be introduced at around age 6 months. However, some medical conditions may require exclusive formula feeding also after 6 months (e.g., those who need to be tube fed). Therefore, formula has been considered as full diet for ages 0-12 months, and as supplementary diet for ages 6-12 months.

According to the directions for usage of the two products Pepti Syneo and Neocate Syneo (ToR 2), "the quantity of feed and the dilution should be determined by a clinician or dietitian and is dependent on the age, body weight and medical condition of the infant" (Nutricia, 2023). VKM has based the quantity of feed on reference values for infants' energy requirement and the standard dilution given in the current product application. VKM has also included an enrichment scenario as health professionals may use a more concentrated formula for infants with poor weight gain or increased energy needs. VKM's enrichment scenario is based on the Norwegian national guideline for newborn medicine (Moltu et al., 2022).

VKM's estimation process is outlined below, and the input data for each step is given in Table 4.2-1. VKM has estimated exposure to *B. breve* M16-V using the concentration in the product Pepti Syneo as ready-to-feed solution. The resulting exposure values will be compared to those found in the literature review of potential health risk. All calculations are available in a supplementary tile to this report at vkm.no. The applicant has stated that the target intake is 10^9-10^{10} cfu per day of *B. breve* M-16V.

4.2 Process and input data

The process of the exposure estimation is outlined below:

1) Select reference values for energy requirement per kg body weight, by age and gender.
2) Select reference values for weight-for-age (low, median, high) and gender.
3) Select value for energy content of formula (Pepti Syneo and Neocate Syneo) per 100 mL ready-to-feed solution.
4) Calculate the amount of formula (ready-to-feed solution or dry weight) needed as full diet to meet the energy requirements.
5) Select the concentration of *B. Breve* M-16V in the formula (cfu per 100 mL ready-to-feed solution or per 1-gram dry weight)
6) Calculate daily exposure to *B. breve* M16-V from formula as full diet
7) Estimate daily exposure to *B. breve* M16-V in enrichment scenario for the 3rd and 50th, weight-for-age percentile, adding more formula powder to the standard solution
8) Estimate the amount of formula consumed as supplementary diet for ages 6-12 months, implying a lower formula intake than as full diet
9) Estimate daily exposure to *B. breve* M16-V from formula as supplementary diet

An overview of the input data is given in Table 4.2-1 with explanations provided below the table.

Table 4.2-1. Data used in exposure estimations.

Step	Input data	Source	Specification	Reference
1	Reference values, infant energy requirements	Norwegian national guideline on infant nutrition	Mean values for 1, 3, 6, 12 months for boys and girls	Norwegian Directorate of Health (HDIR, 2017)
2	References values, weight-for-age	WHO child growth standards	3rd, 50th and 97th percentiles for boys and girls	WHO, 2006
3	Formula, energy content, Pepti Syneo	Pepti Syneo data card	100 mL ready formula: 276 KJ (66 kcal)	Nutricia, 2023
4	Calculation of formula intake using steps 1-3			
5	Formula concentration of *B. Breve* M-16V	From applicant	100 mL ready formula: 1.47×10^9 cfu	Application, clinical documentation
5		From applicant	1 gram powder: 10^7–10^8 cfu	Application, VKM checklist
5		From applicant	14.4 g powder needed + 90 mL water to achieve 100 mL providing 14.4×10^7–10^8 cfu /100 mL ready formula	Application, VKM checklist
6	Calculation of exposure using intake (step 4) and *B. Breve* concentration (step 5)		Age 1, 3, 6, 12 months	
7	Formula enrichment scenarios	National guideline for newborn medicine, chapter 14.4-14.5	Add 15% to 30% more formula powder (0.5-1 scoop) to standard solution	Moltu et al., 2022.
7	Formula enrichment, Pepti Syneo	Pepti Syneo data card	1 level scoop = 4.6 g powder	Nutricia, 2023.
8	Volume of human milk intake during complementary feeding	Systematic review	6 months: 107 mL/kg/day, 12 months: 61 mL/kg/day	Rios-Leyvraz et al., 2023
9	Calculation of exposure using intake (step 8) and *B. Breve* concentration (step 5)			

Energy requirements: In the national guideline on infant nutrition from the Norwegian Directorate of Health (HDIR, 2017), the references values for infant energy requirements are given for ages 1, 3, 6, 12 months by gender. The values (means without a measure of spread) are the same as the requirements given in the more recent Nordic Nutrition Recommendations, NNR (Blomhoff et al., 2023), but because the NNR only provide values for ages 6 and 12 months, VKM used the guideline on infant nutrition from 2017.

Weight-for-age: VKM has taken the 3rd, 50th, and 97th percentiles of the WHO growth standard (WHO, 2006) to represent low, median, and high body weights for the ages 1, 3, 6, 12 months. VKM selected percentiles for interpretability over Z-scores. The enrichment scenario was performed for infants in the 3rd and 50th percentile who receive formula as a full diet.

Formula composition (energy, probiotic, and prebiotics): There is a slight difference in the energy content of the two products Pepti Syneo and Neocate Syneo (ToR 2). Pepti Syneo has been emphasized by the applicant and has been used in main calculations. The concentration of *B. Breve*

M-16V is assumed to be similar. Exposure calculations are not performed for the prebiotic content. According to the applicant, Pepti Syneo contains around 90% short-chain galacto-oligosaccharides (scGOS) and 10% long-chain fructo-oligosaccharides (lcFOS), whereas Neocate Syneo contains 90% short-chain fructo-oligosaccharides (scFOS) and 10% lcFOS. The total prebiotic content is around 6.8 g/L. In the application information, there is a small discrepancy between the reported concentration of *B. breve* M-16V in ready formula (1.47×10^9 cfu per 100 mL) and the recipe given in the application stating that 14.4 g powder should be mixed with 90 ml of water to achieve a 100 ml ready to eat portion providing 14.4×10^7-10^8 cfu (or 1.44×10^8-10^9 cfu) per 100 ml ready formula. The discrepancy was not clarified by the applicant, thus VKM decided to use the highest value in the calculations (1.47×10^9 cfu per 100 mL), which is also frequently reported in the literature (Table 3.12.4-2).

Enriched infant formula: The Norwegian national guide to newborn medicine (Moltu et al., 2022) suggest that milk can be enriched by adding 15-30% more powder to the standard solution corresponding to 0.5 to 1 scoop (using the scoop provided with the formula). VKM has estimated the dose of *B. breve M16*-V from enriching the total daily volume of formula by adding 0.5 or 1 scoop of Pepti Syneo, corresponding to 2.3 (16%) or 4.6 (32%) grams of extra formula powder per 90 mL water to the standard solution.

4.3 Resulting exposure values

The resulting values for exposure to *B. breve* M16-V are shown below for different ages and weight percentiles, by gender, for formula as full diet (Table 4.3-1), formula as full diet enriched with additional powder (Table 4.3-2), and formula as supplementary diet (Table 4.3-3) using concentrations in Pepti Syneo and ready-to-feed formula.

Table 4.3-1. Estimated daily exposure to *B. breve* M16-V (10^9 cfu/day) from formula as full diet, by gender and age for different weight percentiles, P (3, 50, 97).

	Boys: exposure (109 cfu/day) by weight percentiles			Boys: exposure (109 cfu/day) by weight percentiles		
Age (months)	**3rd P**	**50th P**	**97th P**	**3rd P**	**50th P**	**97th P**
1	0.88	1.14	1.45	0.77	1.02	1.32
3	1.11	1.40	1.72	0.99	1.25	1.60
6	1.15	1.43	1.75	1.06	1.33	1.68
12	1.41	1.73	2.13	1.28	1.60	2.01

Table 4.3-2. Estimated daily exposure to *B. breve* M16-V (10^9 cfu/day) from formula as full diet enriched with additional powder (0.5 or 1 scoop) by gender and age for different weight percentiles, P (3, 50).

	Boys: exposure (109 cfu/day) by enrichment and weight percentiles				Girls: exposure (109 cfu/day) by enrichment and weight percentiles			
	0.5 scoop (2.3 g) added		**1 scoop (4.6 g) added**		**0.5 scoop (2.3 g) added**		**1 scoop (4.6 g) added**	
Age (months)	**3rd P**	**50th P**	**3rd P**	**50th P**	**3rd P**	**50th P**	**3rd P**	**50th P**
1	1.02	1.31	1.15	1.49	0.89	1.18	1.02	1.32
3	1.29	1.61	1.46	1.83	1.15	1.44	1.30	2.61
6	1.34	1.64	1.52	1.87	1.37	1.72	1.56	1.93
12	1.63	2.00	1.85	2.27	1.48	1.85	1.68	2.07

Table 4.3-3. Estimated daily exposure of B. breve M16-V (10^9 cfu/day) from formula as supplementary diet, by gender and age for different weight percentiles, P (3, 50, 97).

	Boys: exposure (10^9 cfu/day) by weight percentiles			Girls: exposure (10^9 cfu/day) by weight percentiles		
Age (months)	**3^{rd} P**	**50^{th} P**	**97^{th} P**	**3^{rd} P**	**50^{th} P**	**97^{th} P**
6	1.00	1.24	1.53	0.91	1.15	1.45
12	0.70	0.86	1.06	0.65	0.81	1.01

Chatchatee et al., 2022 reported that the mean intake volumes of study formula at ages 6 and 12 months were 602 mL and 538 mL, respectively, when given as supplementary diet (gender or body weight not specified). Given boys at median weight-for-age, and the concentration of *B. breve* M-16V of 1.47×10^9 cfu/100 mL, these volumes result in an exposure of 0.88 and 0.79×10^9 cfu per day at 6 and 12 months, respectively. VKM´s calculations of exposure to *B. breve* M-16V when formula is given as supplementary diet is in the range from 0.65×10^9 to 1.53×10^9 cfu/day (Table 4.3-3).

4.4 Scenarios in GRAS report and application to NFSA

The GRAS status of *B. Breve* M-16V for intended use as an addition to powdered amino acid-based exempt term infant formulas (FDA, 2013c), applies to the addition of 10^8 colony forming units (cfu) per gram of powdered formulation to produce a target intake range of $10^9 – 10^{10}$ cfu per day. The intake of a one month- and six month-old infant was calculated to be 9.9×10^9 and 1.35×10^{10} cfu of *B. breve* M-16V per day, respectively based on 10^8 cfu per gram of powdered formula, that infant formulas in the US market typically provide 0.67 kcal/mL, that formula was the sole source of nutrition, reconstituted at 14.1 g/100 mL and a caloric density of 0.67 kcal/mL, and that the caloric requirements of one month-old and a six month-old infant are 472 kcal/day and 645 kcal/day, respectively. The energy requirements were from the Institute of Medicine (US) Panel on Macronutrients and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes from 2005.

The current application to the NFSA includes a scenario for older infants (6-12 months) based on the highest observed mean daily energy intake of 980 kcal (EFSA, 2014). For this age interval, the contribution of follow-on formula towards 980 kcal is estimated to be between 60-40%, approximating a maximum (60%) intake of 900 mL and 1.3×10^9 -10^{10} cfu per 900 mL according to the applicant.

4.5 Summary of exposure

The highest resulting exposure to *B. breve* M16-V from intake of infant formula is expected to occur if boys aged 12 months in the highest weight-for-age percentile (97^{th}) receive formula as a full diet (requires 14,5 dl formula per day). The exposure in this group, 2.13×10^9 cfu per day, is similar to the target intake reported by the applicant (10^9-10^{10} cfu per day without higher precision). In general, exposure estimates in girls will be slightly lower than in boys, due to lower weight-for-age.

Formula enrichment with one additional scoop of powder for infants (boys and girls) age 0-12 months in the median percentile (50^{th}) of weight-for-age, leads to exposure in the range 1.32 to 2.27×10^9 cfu per day and is slightly higher than the highest value without enrichment (boys in the 97^{th} percentile who receive formula as full feed).

Use of formula as supplementary diet leads to a lower exposure than formula as full diet. A potential effect of complementary foods on the activity of *B. breve* M16-V is considered an uncertainty (see Chapter 6). Underlying assumptions of all calculations are that infants are fed based on their energy requirements and that all the formula is consumed.

5 Risk characterization

This risk characterization of *B. Breve* M-16 in infant formula comprises three elements; the safety characteristics of *B. breve* M-16 as such, primarily based on GRAS from 2013, and health risk characterizations of various outcomes based on human studies in infants from a systematic literature review conducted by VKM, and finally VKM´s exposure calculations to *B. breve* M-16V.

5.1 Safety characteristics of *B. breve* M-16V

For characterization of risk related to the consumption of *B. breve* M6-V, VKM has evaluated the following genetic/genomic and phenotypic properties of the strain: antimicrobial resistance patterns and whether resistance genes are located on, or associated with, mobile genetic elements (plasmids, transposons or integrons); virulence factors; specific metabolites or metabolic activities (production of D-lactate, biogenic amines, ammonia production, bile salt deconjugation, azoreductase or nitroreductase activity); haemolytic activity; effects on platelet aggregation or viability; adherence to mucus and/or human epithelial cells and cell lines, and toxin production, as well as oral toxicity in animal models.

The submitted documentation from the applicant did not provide significant additional information beyond the documentation supporting the regulatory status of GRAS and QPS. The documentation did not indicate that the strain possesses any pathogenic or toxic properties. Furthermore, there was no indication that it may contribute significantly to spreading antimicrobial resistance genes to other bacterial taxa.

5.2 Health risk characterization based on studies in infants

The health risk characterization is based on 14 publications on 13 studies with infant formula containing *B. breve* M-16V: 12 RCTs and one observational study. The studies comprised a total of 836 infants who received formula with added *B. breve* M-16V, of which 491 infants in four studies were described as healthy and 345 infants in eight studies had cow´s milk allergy or atopic dermatitis. Age at inclusion varied from birth to three years, and the duration of the intervention was six weeks to 12 months (median 16 weeks). The infant formulas contained *B. breve* M-16V in a concentration range of 1×10^6 to 1.47×10^9 cfu/100 mL. The formula was given as a full diet in eight studies that included 478 infants and as a supplementary diet in eight studies that enrolled 358 infants.

Additionally, the health risk characterization is based on two reviews and four primary studies with enteral feeding with *B. breve* M-16V in premature infants. A substantial part of the literature on *B. breve* M-16V is studies of infants born prematurely that receive enteral feeding. VKM considered this literature as relevant for the risk assessment as supportive evidence since results on safety may be valid for infants born at term.

As requested in the ToR, VKM has assessed the risk of consumption of *B. breve* M-16V given as a supplement in infant formula. Most of these formulas contained a prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) of different sources (inulin, chicory, raffinose). Prebiotics may have an indirect effect on the microbiome as substrate for the bacteria. Furthermore, probiotics can have an impact on gastrointestinal symptoms. VKM has not been able to separate the effects of *B. breve* M-16V from the potential effects of prebiotics since they co-exist in the synbiotic formula that is used in most of the studies. Furthermore, specific hypoallergenic formulas given to infants with cow´s milk allergy consist of amino acids or extensively hydrolysed protein. VKM has not considered the modified protein source of the formulas in the health risk characterization.

The introduction of complementary foods, typically at the age of four to six months, will result in lower intake of study formula and lower intake of *B. breve* M-16V. However, it is uncertain how the activity of *B. breve* M-16V may change in the presence of complementary foods acting as prebiotics such as dietary fibres.

VKM´s health risk characterization is summarized by the following outcomes reported in studies of *B. breve* M-16V in infants: adverse events, infant growth, gastrointestinal-, skin- and airway symptoms (formula studies) and premature birth complications (enteral feeding). Health risks were then evaluated as short-term or long-term. VKM defined short-term as health risk occurring during intake, and long-term as risk occurring after cessation of intake of *B. breve* M-16V.

5.2.1 Adverse events

As previously stated, VKM adopted the distinction between “adverse events” (AE) and “adverse effects” from the Cochrane Handbook for Systematic Reviews of Interventions (see glossary section) stating that for an adverse event to be considered an adverse effect, there has to be at least a reasonable possibility of a causal relation between the intervention and the event.

The risk characterization regarding infant growth was based on ten studies where 736 infants (491 healthy infants) received test formula with *B. breve* M-16V. The studies comprised a wide range of definitions of AE. No studies reported higher prevalence of serious AE in test group receiving *B. breve* M16-V than in control group. On the contrary, one study reported more serious AE in the control group than in the test group. Three studies reported significantly higher prevalence of any AE or mild AE in test group than in control group. Study authors concluded that the events had no relation to the study product. Only one in three studies provided an explanation for this conclusion. Remaining studies reported that there was no difference between study groups or that the prevalence of AE was higher in control than in test group.

Overall, VKM did not identify any consistent differences suggestive of adverse effects related to intake of formula containing *B. Breve* M-16V. VKM has moderate confidence in the body of evidence on adverse events (section 3.12.11). Downgrading of the confidence is due to imprecision and potential for publication bias.

5.2.2 Infant growth

The risk characterization regarding infant growth was based on nine studies where 706 infants (491 healthy infants) received test formula with *B. breve* M-16V. Growth was within expected ranges for age in all infants that consumed *B. breve* M-16V. Abrahamse-Berkeveld et al., 2016 reported slightly lower absolute length in infants who had received *B. breve* M-16-V than in control infants at all time points in the PP population, but not in the ITT population. Still, the test infants grew in accordance with WHO standards. The authors could not find any good explanations for this effect. The 105 infants in this study were healthy and received formula as a full diet. In infancy and early childhood poor ponderal growth usually precedes linear growth. Here, there was no impact on either absolute weight or weight Z-scores. The observed effect was probably a chance finding.

VKM did not identify any risk of disturbed growth in infants receiving *B. breve* M16-V. VKM has moderate confidence in the body of evidence of infant growth (section 3.12.11). Downgrading of evidence is due to the potential for publication bias.

5.2.3 Gastrointestinal (GI) symptoms

The risk characterization regarding GI-symptoms was based on eight studies where 656 infants (491 healthy infants) received test formula with *B. breve* M-16V. Gastrointestinal symptoms may have serious impact on infants if diarrhoea results in dehydration and electrolyte disturbances. There were no reports of such severity. VKM did not identify any risk of consumption of *B. breve* M-16V in synbiotic formula or unintended or worsening effects of GI symptoms.

Five of the eight studies reporting on GI symptoms had low risk of bias (Tier 1) according to OHAT method for screening risk of bias. Tree studies were placed in Tier 2 and none in Tier 3 (section 3.12.5).

5.2.4 Skin symptoms

The risk characterization regarding skin symptoms was based on eight studies where 420 infants (105 healthy infants/without atopic dermatitis) received test formula with *B. breve* M-16V. There were no reports of an increase in skin symptoms related to the intervention. VKM did not identify any risk of unintended or worsening effects of skin symptoms in infants consuming *B. breve* M-16V in synbiotic formula.

Five of the eight studies reporting on skin symptoms had low risk of bias (Tier 1) according to OHAT method for screening risk of bias. Two studies were placed in Tier 2 and one in Tier 3 (section 3.12.5).

5.2.5 Airway symptoms

The risk characterization regarding airway symptoms was based on five studies where 314 infants (105 healthy infants) received test formula with *B. breve* M-16V. There were no reports of increased airways symptoms related to the intervention. VKM did not identify any risk of unintended or worsening effects of airway symptoms in infants consuming *B. breve* M-16V in synbiotic formula.

Four of five studies reporting on airway symptoms received Tier 1 (low risk of bias) according to OHAT method for screening risk of bias, and one study received Tier 2 (See section 3.12.5).

5.2.6 Enteral feeding for premature birth complications

Two systematic review studies did not report any unintended effects or worsening of complications in premature infants receiving *B. breve* M-16V as part of enteral feeding. For outcomes defined to be of critical importance in one review: stage ≥2 NEC, all-cause mortality, and late-onset sepsis, the overall confidence in the evidence was graded "very low" for each outcome by the study authors due to a scarcity of studies. Another review of probiotic sepsis from 2022 found no results or case reports on *B. breve* M-16V. The risk of bias was graded "low" by VKM (section 3.12.3). Thus, VKM has confidence in the methodology of the review and the null finding. However, the total number of subjects investigated was unknown.

One primary study on enteral feeding published after the review from 2018 supported the results in the previous review of stage ≥2 NEC, late-onset sepsis, weight, and length Z-scores. Another study (Priyadarshi et al. 2021) reported no findings of probiotic induced sepsis and thus supports the null findings in Kulkarni et al., 2022. The other primary studies assessed other outcomes. One study was a follow-up of preterm infants for neuropsychological development 3-5 years after the intervention with *B. breve* M-16V (Agrawal et al., 2020). No negative effects were reported. The last study (Indrio et al., 2022) assessed risk factors for hyperglycemia among extremely low birth weight infants (<1000 g). Increased risk of hyperglycemia was reported for *B. breve* M-16V, but pointed to dextrin used as an excipient as a potential mechanism, not the bacteria per se.

All but two of the outcomes received Tier 3 according to OHAT and indicate high risk of bias. Studies on hyperglycemia and probiotic induced sepsis were classified in Tier 2.

5.2.7 Short term and long-term health risks

Most studies assessed health risks during the intervention period (median 16 weeks). The longest duration of exposure to *B. breve* M-16V was 12 months, found in one study (Chatchatee et al., 2022). VKM did not identify any health risk of feeding infants with cow´s milk allergy a synbiotic formula containing 1.47×10^9 cfu/100 mL of *B. breve* M-16V. Based on this study and the above findings from the literature in infants, there was no short-term health risk identified due to the consumption of synbiotics.

VKM's literature search identified only two follow-up studies that assessed disease development after the intervention period with *B. breve* M-16V was terminated. One study of formula feeding in infants with atopic dermatitis had one-year follow up for asthma-like symptoms (van der Aa et al., 2011), and one study of enteral feeding of premature infants had 3 to 5 years of follow-up for neuropsychological outcomes (Agrawal et al., 2020). There were no reports of negative effects on disease development in any of the two studies. However, the study with the longest follow-up was limited by substantial loss of study subjects during follow-up, and the study was in the Tier 2 for risk of bias according to the OHAT method, indicating high risk of bias. Thus, VKM ascertained that studies of long-term health risks of *B. breve* M-16V are very limited.

5.3 Specific assessment related to PEPTI SYNEOTM and NEOCATE SYNEOTM

b. PEPTI SYNEO™ as full diet from 0-6 months
c. PEPTI SYNEO™ as supplementary diet from 6-12 months
d. NEOCATE SYNEO™ as full diet from 0-12 months

The evidence of the specific risk characterization related to PEPTI SYNEO™ and NEOCATE SYNEO™ is based on 11 publications from eight studies that comprised 283 infants with cow´s milk allergy or hypersensitivity and 62 infants with atopic dermatitis, who received study formula with *B. breve* M-16V from 7.5×10^8 to 1.47×10^9 cfu per 100 mL. The age of the infants at inclusion was from birth to three years, and the duration of the intervention ranged from four weeks to 12 months. The infants were given either amino-acid-based or extensively hydrolysed formula. Three of the studies specified the formulas as Neocate Infant DHA, Neocate LCP or Neo-Syn. The formula was given as a full diet in 46 infants and as a supplementary diet in 299 infants. VKM did not identify any risk of adverse effects, growth disturbances, or unintended or worsening effects on GI, skin, and airway symptoms.

5.4 Exposure

Table 5.4.1 shows a summary of VKM's exposure scenarios (full diet or supplementary diet) compared with other exposure estimates reported in the research literature or provided by the applicant or in GRAS documentation. VKM´s calculations resulted in slightly higher exposure of *B. breve* M-16V in colony forming units (cfu) per day as compared to the amount derived from the only study that reported volume intake of formula at 6 and 12 months. VKM´s calculations are based on energy requirements by gender, age, WHO growth standards and the assumption that the infants 0-12 months are exclusively formula fed. Normally the intake of formula decreases by the age of four to six months when complementary foods are gradually introduced. Only in certain situations where infants are dependent on total enteral nutrition will the formula be the sole source of nutrition. Likewise, the GRAS calculations are based on energy requirement at different ages with the assumption that the infant formula is the sole nutrition. The amounts of *B. breve* M-16V derived from the literature are based on observed mean intake of infant formula as supplementary diet at different ages during the study periods and are therefore lower than both VKM´s and GRAS´s theoretical calculations. However, VKM´s calculations of exposure when formula is given as a supplementary diet ($0.91\text{-}1.53 \times 10^9$ cfu/day at 6 months and $0.65\text{-}1.06 \times 10^9$ cfu/day at 12 months) are comparable to the exposure from the literature (0.88 and 0.79×10^9 cfu/day at 6 and 12 months). The applicant´s calculation is based on an observed maximum formula intake in infants at six and 12 months and matches the exposure calculated by VKM. Finally, both the applicant´s calculations and the exposure ranges estimated by VKM are within the theoretical exposure given in GRAS 455.

Table 5.4-1. Summary of exposure calculations (section 4.3) and scenarios (section 4.4) for *B. breve* M-16V (10^9 cfu/day[1]).

Age (months)	VKM´s calculations full diet	VKM´s calculations supplementary diet	VKM's calculations Chatchatee et al., 2022	Applicant's scenario	GRAS scenario
1	0.77-1.45				9.9
3	0.99-1.72				
6	1.06-1.76	0.91-1.53	0.88	1.3	13.5
12	1.28-2.13	0.65-1.06	0.79	1.3	

[1] All numbers in table given in 10^9cfu, colony forming units, per day.

5.5 Summary of risk characterization

The risk characterization is based on three elements: the safety characteristics of *B. breve* M-16V, the literature review of human studies in infants, and the exposure calculations.

VKM considers that the documentation on safety characteristics of *B. breve* M-16V (phenotypic and genetic/genomic properties) did not indicate that the strain possesses any pathogenic properties or that it can pose a significant health risk to humans.

VKM´s systematic review of 13 human studies in 820 infants receiving *B. breve* M-16V did not reveal any health risks of consuming infant formula with *B. breve* M-16V in concentrations of 1 × 10exp6 to 3×10^9 cfu/100 mL as full diet or supplementary diet for six weeks to 12 months. No health risks were identified in 439 healthy infants or 381 infants with cow´s milk allergy or atopic dermatitis. VKM did not identify any serious adverse effects or disturbed infant growth. No unintended or worsening effects of GI, skin, or airways symptoms were identified.

The confidence in the body of evidence was evaluated for adverse events and infant growth. The evaluation resulted in downgrading from high to moderate confidence due to serious imprecision of adverse events and the potential for publication bias for both outcomes.

VKM´s exposure calculations to *B. breve* M-16V show that there is no deviation from the exposure of *B. breve* M-16V as described in the literature when formula intake normally decreases from six months of age. Theoretically, a high intake of formula, when given as a full diet for up to 12 months, such as in the VKM calculation of exposure, the amount of *B. breve* M-16V represents a lower exposure as compared to the amounts given in the literature and compared to the exposure calculated by GRAS 455.

6 Uncertainty

EFSA recommends that assessments identify areas of uncertainty and state clearly their subsequent impact on the overall assessment outcome for the purpose of clarity and transparency in risk assessment processes. Additionally, this is critical in the subsequent selection of risk management options (EFSA et al., 2018). The degree of confidence in the final risk estimation depends on the variability, uncertainty, and assumptions identified in all the previous steps. Discrimination between uncertainty and variability is important in the subsequent selection of risk management options. Biological variation includes, for instance, the differences in resistance levels that exist in microbiological populations over time and between hosts and environments, including random fluctuations (FAO, 1999).

Several uncertainties related to *B. Breve* M-16V have been identified in this assessment. Most of these uncertainties are qualitative and may overlap with data gaps:

- Long-term health effects of exposure to *B. breve* M16-V in infancy
- Possible microbiome alteration in infancy due to consumption of *B. breve* M16-V
- The potential for long-term colonization of *B. breve* M16-V
- Physiological impact of *B. breve* M16-V on the microbiota in hosts
- How the activity of *B. breve* M-16V may change in the presence of complementary foods acting as prebiotics (e.g. dietary fibres)

VKM has not been able to conclude on the long-term health risk of exposure to *B. breve* M-16V during infancy due to lack of studies (see Data gaps). In newborn children, establishment of the gastrointestinal microbiome is considered an essential developmental process. It has been hypothesized that in infants probiotic microorganisms could become primary colonizers that remain long-term, perhaps even for life (FAO/WHO 2001), with largely unknown health consequences. The ability to cause long-term health effects may depend on the potential of a specific probiotic bacteria to colonize the host gastrointestinal mucosal surface (transiently or permanently), the requirement for and availability of nutrients, and ability to interact with the indigenous microbiota or microbiome, and the role of the microbiome on the immune system. As described in the study protocol, the initial scoping search for literature indicated long-standing debates and uncertainties related to these proposed mechanisms of action of probiotic bacteria. This is the background for the study delimitation (section 1.2) of not doing a formal review of mechanistic evidence, as VKM expected inconclusive results. There is a body of literature describing the influence of multiple factors on the development of the gut microbiome, including prenatal environment, host genetics, mode of delivery, gestational age, type of infant feeding (breastfeeding or formula feeding), antibiotics use, and environmental factors (Tamburini et al. 2016; Milani et al., 2017) but the relative importance remains debated. Differences in the gut microbiome of breast-fed and formula fed infants, has motivated food manufacturers to add probiotics to infant formula, to compensate for the bacteria transmitted by breast feeding. Disruptions in the development of the gut microbiome have been linked to health issues later in life, such as asthma and allergies, irritable bowel disease, overweight/obesity, and diabetes (Tamburini et al., 2016) but currently it seems unknown if infant feeding with probiotic bacteria, and more specifically *B. breve* M-16V as a single strain in doses 10^9-10^{10} cfu per day, could prevent or cause such disruptions.

7 Conclusions (with answers to the terms of reference)

The risk characterization based on properties of *B. breve* M-16V, a literature review of studies in infants and exposure calculations does not reveal any health risks of infant formula containing *B. breve* M-16V. The bacterial strain in question is quite well studied, both from a phenotypic/genetic perspective and in terms of potential impact on infant health. However, there is a paucity of data evaluating potential long-term effects, for example on the development of the gastrointestinal microbiome and the immune system, and later implications for health. There is also a lack of studies on infant formula that are independent (funding and/or authorship) of the manufacturer of *B. Breve* M-16V or formula. This is not the case for studies on enteral feeding.

The Norwegian Food Safety Authority asked VKM to answer the following questions:

1. Are there any health risks of giving infant formulas containing *Bifidobacterium breve M-16V* to infants 0-12 months:
    a. As a full diet
    b. As a supplementary diet

Answer to The Norwegian Food Safety Authority

VKM concludes that the current documentation on safety characteristics of *B. breve* M-16V does not indicate that the strain possesses any unwanted phenotypic or genetic/genomic properties.

VKM´s systematic review of 13 human studies in a total of 1580 infants did not indicate short term health risks of consuming infant formula with *B. breve* M-16V. Of 1580 infants, 836 were given *B. breve* M-16V in concentrations of 1×10^6 to 1.47×10^9 cfu/100 mL formula as full diet or supplementary diet for six weeks to 12 months. No health risks were identified in either healthy infants or in infants with cow´s milk allergy or atopic dermatitis. VKM did not identify any serious adverse effects or disturbed infant growth. No unintended or worsening effects of GI, skin or airway symptoms were identified.

VKM´s exposure calculations to *B. breve* M-16V are consistent with exposure to *B. breve* M-16V as described in the literature (based on one study reporting the volume of formula intake) when formula is given as supplementary diet. A higher intake of formula, when given as a full diet for up to 12 months, results in higher intake of *B. breve* M-16V than the amounts given in this single study but is within the amounts estimated in the GRAS scenario.

2. Are there any health risks of giving two specific hypoallergenic infant formulas containing *Bifidobacterium breve M-16V* (marketed as foods for special medical purposes) to infants with cow´s milk allergy and/or other food allergies and related allergic conditions:
    a. PEPTI SYNEO™ as full diet from 0-6 months
    b. PEPTI SYNEO™ as supplementary diet from 6-12 months
    c. NEOCATE SYNEO™ as full diet from 0-12 months

Answer to The Norwegian Food Safety Authority

VKM has the same answer to the product specific question; no risks were identified. The conclusion here is based on fewer studies and fewer study infants. VKM did not identify any risks of adverse effects, growth disturbances, or worsening of gastrointestinal-, skin-, or airway symptoms from the eight studies where 345 infants (aged 0-3 years) with cow´s milk allergy or atopic dermatitis were fed specific hypoallergenic infant formulas containing *B. breve M-16V,* as a full diet or as a supplementary diet for periods of 8 weeks to 12 months.

Overall, VKM has not identified health risks of consuming general or allergy specific infant formula containing *B. breve* M-16V among infants 0-12 months. VKM has moderate confidence in the body of evidence for the outcomes described in the literature that VKM considered to have high clinical relevance, adverse events and infant growth. VKM has not been able to assess potential long term health effects due to only two studies on different health outcomes.

8 Data gaps

- A major data gap in this evaluation concerns potential long-term effects of *B. breve* M-16V administration during infancy. There is little research on how infant feeding with probiotics may impact important developmental processes, like immune function, and development of immune mediated diseases later in life.
- Most of the studies in the current risk assessment are primarily designed to investigate potential beneficial effects of consumption of *B. breve* M-16V. Thus, adverse events and safety parameters are often defined and treated as secondary outcomes.
- Only two studies on infant formula had a design where the effect of *B. breve* M-16V could be separated from that of prebiotics.
- There is a lack of studies on infant formula with *B. breve* M-16V that have been conducted independently of the manufacturers.
- Another data gap concerns the presence of mobile genetic elements (MGE) in the genome of *B. breve* M-16V. While the applicant documented the absence of plasmids, there was no attempt to do the same for elements like integrons and insertion sequences. This could, in part, be achieved through a database search like what was done for antibiotic resistance and virulence genes. On the other hand, gene databases of this kind are far from complete, and for MGEs it can be expected that large numbers of marker sequences are absent from databases simply because they are yet to be discovered. Thus, even a negative result from a database search would in no way constitute evidence of absence. Furthermore, most bacterial genomes can be expected to contain MGEs of some kind (e.g. phage sequences) without this being problematic for the safety profile of the carrier species.

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10 Appendixes and list of supplementary files

10.1 Appendix: literature search strategy

BIFIDOBACTERIUM BREVE M-16V IN INFANT FORMULA

Contact person:	Christine Louise Parr
Search:	Astrid Nøstberg
Peer review:	Bente Foss
Duplicate control in EndNote:	Before duplicate control: 2115
	After duplicate control: 1513

The research question the literature search is intended to answer			
Are there any health risks of giving infant formula containing Bifidobacterium breve M-16V to infants 0-12 months?			
The research question according to the PICO formula			
Population	**Intervention**	**Comparison**	**Outcome**
Infants (age 0-12 months) at the time of intervention/exposure	Infant formula containing the probiotic bacteria Bifidobacterium breve M-16V	Infant formula without probiotic bacteria	Health risks

Database: Ovid MEDLINE(R) and Epub Ahead of Print, In-Process, In-Data-Review & Other Non-Indexed Citations, Daily and Versions 1946 to August 23, 2023

Date: 23.08.2023

Number of hits: 718

#	Searches	Results
1	exp Infant/ or exp Pediatrics/ or Child Development/	1326118
2	(pediatric* or paediatric* or perinatology or neonatology or infant? or suckling? or newborn? or "new born?" or neonat* or "neo nat*" or toddler*).tw,kf.	1214349
3	(((age or aged) adj ("1" or one or "2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*")) or (("1" or one) adj ("year* old" or "yr* old")) or (("2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*") adj old)).tw,kf.	97009
4	or/1-3	1947143
5	Infant Formula/ or Probiotics/ or Prebiotics/ or Synbiotics/	32356
6	((infant or baby or feeding) adj3 (formula or formulas or formulae?)).tw,kf.	9763
7	((formula or formulas or formulae? or artificial or synthetic) adj3 milk).tw,kf.	5525
8	(("amino acid" adj3 (formula or formulas or formulae?)) or (aminoacid adj3 (formula or formulas or formulae?))).tw,kf.	470
9	(probiotic? or prebiotic? or synbiotic? or "AAF-S").tw,kf.	47950
10	or/5-9	65926
11	Bifidobacterium breve/ or Bifidobacterium/	6363
12	(breve adj3 ("M 16V" or M16V or "M16 V")).tw,kf.	73

13	11 or 12	6382
14	4 and 10 and 13	775
15	Animals/ not (animals/ and humans/)	5114509
16	14 not 15	718

Database: Embase <1974 to 2023 August 23>
Date: 24.08.2023
Number of hits: 1036

#	Searches	Results
1	exp infant/ or pediatrics/ or toddler/ or child development/	1249759
2	(pediatric* or paediatric* or perinatology or neonatology or infant? or suckling? or newborn? or "new born?" or neonat* or "neo nat*" or toddler*).tw,kf.	1573617
3	(((age or aged) adj ("1" or one or "2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*")) or (("1" or one) adj ("year* old" or "yr* old")) or (("2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*") adj old)).tw,kf.	135922
4	or/1-3	2161738
5	artificial milk/ or probiotic agent/ or prebiotic agent/ or synbiotic agent/	74638
6	((infant or baby or feeding) adj3 (formula or formulas or formulae?)).tw,kf.	12328
7	((formula or formulas or formulae? or artificial or synthetic) adj3 milk).tw,kf.	7114
8	(("amino acid" adj3 (formula or formulas or formulae?)) or (aminoacid adj3 (formula or formulas or formulae?))).tw,kf.	872
9	(probiotic? or prebiotic? or synbiotic? or "AAF-S").tw,kf.	58405
10	or/5-9	95192
11	Bifidobacterium breve/ or Bifidobacterium/	15518
12	(breve adj3 ("M 16V" or M16V or "M16 V")).tw,kf.	126
13	11 or 12	15533
14	4 and 10 and 13	1556
15	limit 14 to (embase or "preprints (unpublished, non-peer reviewed)")	1175
16	(animal/ or exp nonhuman/ or Animal experiment/) not ((animal/ or exp nonhuman/ or Animal experiment/) and exp human/)	6774825
17	15 not 16	1036

Database: **Web of Science Core Collection**: Science Citation Index Expanded (SCI-EXPANDED) --1987-present, Social Sciences Citation Index (SSCI) --1987-present, Arts & Humanities Citation Index (A&HCI) --1987-present, Emerging Sources Citation Index (ESCI) --2018-present

Date: 24.08.2023

Number of hits: 42

Comment: Exact search has been applied

#	Query	Results
#1	TS=(pediatric* or paediatric* or perinatology or neonatology or infant$ or suckling$ or newborn$ or "new born$" or neonat* or "neo nat*" or toddler*)	1151848
#2	TS=(((age or aged) NEAR/0 ("1" or one or "2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*")) or (("1" or one) NEAR/0 ("year* old" or "yr* old")) or (("2 month*" or "two month*" or "3 month*" or "three month*" or "4 month*" or "four month*" or "5 month*" or "five month*" or "6 month*" or "six month*" or "7 month*" or "seven month*" or "8 month*" or "eight month*" or "9 month*" or "nine month*" or "10 month*" or "ten month*" or "11 month*" or "eleven month*" or "12 month*" or "twelve month*") NEAR/0 old))	101062
#3	#1 OR #2	1217954
#4	TS=((infant or baby or feeding) NEAR/2 (formula or formulas or formulae$))	11855
#5	TS=((formula or formulas or formulae$ or artificial or synthetic) NEAR/2 milk)	5369
#6	TS=(("amino acid" or aminoacid) NEAR/2 (formula or formulas or formulae$))	456
#7	TS=(probiotic$ or prebiotic$ or synbiotic$ or "AAF-S")	70721
#8	#4 OR #5 OR #6 OR #7	84606
#9	TS=(breve NEAR/2 ("M 16V" or M16V or "M16 V"))	74
#10	#3 AND #8 AND #9	56
#11	TS= (("animal" or "animals" or "canine*" or "dog" or "dogs" or "feline" or "hamster*" or "lamb" or "lambs" or "mice" or "monkey" or "monkeys" or "mouse" or "murine" or "pig" or "pigs" or "piglet*" or "porcine" or "primate*" or "rabbit*" or "rats" or "rat" or "rodent*" or "sheep*" or "veterinar*") NOT ("human*" or "patient*"))	3472212
#12	#10 NOT #11	42

Database: Cochrane Database of Systematic Reviews, Issue 8 of 12, August 2023; Cochrane Central Register of Controlled Trials, Issue 7 of 12, July 2023

Date: 24.08.2023

Number of hits: 298 (CDSR: 1; Central: 297)

ID	Search	Hits
#1	[mh Infant]	41901
#2	[mh Pediatrics]	1179
#3	[mh ^"Child Development"]	2472
#4	(pediatric* or paediatric* or perinatology or neonatology or infant? or suckling? or newborn? or "new born" or "new borns" or neonat* or (neo NEXT nat*) or toddler*):ti,ab	99515
#5	(Abrahamse-Berkeveld et al., -#4)	114862
#6	[mh ^"Infant Formula"]	768
#7	[mh ^Probiotics]	2895
#8	[mh ^Prebiotics]	412
#9	[mh ^Synbiotics]	245
#10	((infant or baby or feeding) NEAR/3 (formula or formulas or formulae?)):ti,ab	1853
#11	((formula or formulas or formulae? or artificial or synthetic) NEAR/3 milk):ti,ab	1226
#12	(("amino acid" or aminoacid) NEAR/3 (formula or formulas or formulae?)):ti,ab	147
#13	(probiotic? or prebiotic? or synbiotic? or "AAF-S"):ti,ab	10634
#14	(Burks et al., -#13)	13501

#15	[mh ^"Bifidobacterium breve"]	62
#16	[mh ^Bifidobacterium]	817
#17	(breve NEAR/3 ("M 16V" or M16V or "M16 V")):ti,ab	64
#18	(Koutsoumanis et al., -#17)	919
#19	#5 and #14 and #18	298

Database: Epistemonikos

Date: 24.08.2023

Number of hits: 21

Comment: Simplified search strategy due to the limited search functionality.

Title/abstract: (pediatric* or paediatric* or perinatology or neonatology or infant or infants or suckling* or newborn* or "new born" or "new borns" or neonat* or "neo nat" or "neo nates" or toddler*) AND ("infant formula" or "infant formulas" or "infant formulae" or "infant formulaes" or "baby formula" or "baby formulas" or "baby formulae" or "baby formulaes" or "feeding formula" or "feeding formulas" or "feeding formulae" or "feeding formulaes" or "milk formula" or "milk formulas" or "milk formulae" or "milk formulaes" or "artificial milk" or "synthetic milk" or "amino acid formula" or "aminoacid formula" or "amino acid formulas" or "aminoacid formulas" or "amino acid formulae" or "aminoacid formulae" or "amino acid formulaes" or "aminoacid formulaes" or probiotic or probiotics or prebiotic or prebiotics or synbiotic or synbiotics or "AAF-S") AND ("M 16V" OR "M-16V" OR M16V OR "M16 V" OR "M16-V")

10.2 Appendix: description of application evaluation process

The applicant provided extensive documentation pertaining to the safety of *B. breve* M-16V, the main document being the comprehensive GRAS dossier. In addition, there were several shorter supplementary documents, mainly describing methods used in testing phenotypic and genetic properties of the strain, and a review article outlining the use of the strain in health applications. Importantly, the applicant was required to fill out a check list, made by the VKM, which contained a number of basic questions about safety aspects of *B. breve* M-16V, as well as other information about the product as a whole.

VKM did not find all the applicant's responses satisfactory. In September 2023, VKM prepared additional questions for further clarification. These questions were conveyed to the applicant by the Norwegian Food Safety Authority (NFSA).

In December 2023, the applicant responded in part to our additional questions, but mostly by referring to previously provided documentation. VKM preferred direct answers to the questions, in a single document. This was communicated to the applicant via the Norwegian Food Safety Authority, in January 2024. Since we did not receive any response from the applicant for several months, VKM decided to set a deadline for the applicant, June 1, 2024. If no response were received, the project group would base its evaluation on the information already provided by the applicant.

The Norwegian Food Safety Authority did not receive a response from the applicant until mid-July 2024. Despite the deadline having passed, VKM reviewed the applicant's responses. This was assessed by two members of the group and was presented to the rest of the project group. While little new data was supplied with this response, the applicant had tried to address questions made by the evaluating committee in the check list form. This effort included more detailed reference to documentation that was already available, as well as some additional references. Some questions remained unanswered, such as a systematic identifier of strain *B. breve* M-16V, as well as some details of procedures for ensuring strain consistency. The applicant sources the strain from Morinaga Milk Industries Ltd in Tokyo, Japan, and have apparently been unsuccessful in obtaining this information. The applicant was also not able to provide a rationale for the target dose of viable probiotic bacteria, although this seems a question of secondary importance. Overall, the latest response represented an improvement over previous versions, including a word document addressing several of VKM’s specific questions.

The project group concluded that the applicant's response was, in general, satisfactory.

10.3 Appendix: details of GRAS documentation

The following information sections are from the GRAS-documentations and all references in these sections are cited in the GRAS documentations.

10.3.1 Antimicrobial resistance patterns

According to the GRAS Notice No. 455: *"The absence of antimicrobial resistance genes in B. breve M-16V was determined by searching for genomic sequences with homology to known antibiotic resistance genes found in other strains of bifidobacteria and lactobacilli (Appendix 6 in GRAS). BLASTN analyses showed that the B. breve M-16V genome contains regions of homology with Expect-values greater than 0.075 indicating that the B. breve M-16V genome does not contain regions of significant homology to sequences of known antibiotic resistance genes. A comprehensive table is provided in GRAS appendix 6. Functional analyses were also performed by Morinaga Milk Industry, Danone Research, the University of Ghent, and PROSAFE (Biosafety Assessment of Probiotics used for Human Consumption, a report cited in the GRAS 455 (Table 7). Morinaga compared the sensitivity of B. breve M-16V and the type strain B. breve ATCC 15700 to antimicrobials and found that the minimum inhibitory concentrations (MICs) of the two strains were similar (a difference of < 2- fold) (Xiao et al., 2010). Danone Research found similar MICs using methods described by Klare et al. (2005), which were confirmed by the University of Ghent (Appendix 7A, B, and C; B. breve M-16V referred to Original no. 200). Importantly, all MICs were equal to or less than the cut-off values proposed by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2008 (Panel on Additives and Products or Substances used in Animal Feed, 2008). PROSAFE found that B. breve M-16V MICs were equal to, if not below, the cut-off values established by PROSAFE for B. breve (Appendix 2A and B (B. breve M-16V referred to EU-PS38)).*

To determine whether B. breve M-16V contains plasmids, the bacteria were lysed, and genomic and extra-genomic material was resolved by agarose gel electrophoresis as described by Anderson and McKay (1983). Furthermore, genomic sequencing of B. breve M-16V found no sequences other than those contained within its single chromosome. Together these results demonstrate that B. breve M-16V does not contain plasmids".

10.3.2 Virulence factors

According to the GRAS: "*Pathogenicity: A BLASTP search was performed of open reading frames of the B. breve M16V genome against a range of known virulence factors, although the source of the database is not clearly defined in the GRAS documentation. A number of significant matches were identified. Further analysis showed that these genes also have high confidence homologues in several other Bifidobacterium spp., and their functions in these species cannot be assumed to be virulent. The outcome of this analysis can be said to be inconclusive, but it does not provide evidence of pathogenicity in B. breve M-16V. A comprehensive table of results is provided in GRAS appendix 8A. The applicant also states that a search was conducted against the more comprehensive VFDB, which includes known virulence factors from wider range of genera and phyla, and this search did not produce significant matches. The strain was found to test negative for endotoxin in an in vitro assay. Furthermore, toxicity studies in rats and mice did not indicate adverse effects of B. breve M-16V."*

10.3.3 Specific metabolic activities

10.3.3.1 Production of D-lactate

According to the GRAS *"The production of D- and L-lactic acid isomers by B. breve M-16V, Lactobacillus delbrueckii subsp. bulgaricus NCFB2772, Lactobacillus rhamnosus LW 744, and the type strain B. breve ATCC15700 was determined by enzymatic assays involving D- and L-lactate dehydrogenase (Appendix 4). L. delbrueckii subsp. bulgaricus NCFB2772, L. rhamnosus LW 744 are known producers of D-lactic and L-lactic acid, respectively. B . breve M-16V produced L-lactic acid but no D-lactic acid in a manner like L. rhamnosus LW 744 and B. breve ATCC15700."*

10.3.3.2 Biogenic amines

According to the GRAS *"Biogenic amines are low molecular weight organic bases formed in foods by the microbial decarboxylation of amino acids or by the transamination of aldehydes and ketones by amino acid transaminases. The ingestion of high amounts of biogenic amines can induce facial flushing, sweating, rash, a burning taste in the mouth, diarrhea, cramps, respiratory distress, swelling of the throat, and blurred vision (reviewed in Ladero et al., 2010). To determine whether the genome of B. breve M-16V contains genes capable of conferring amino acid decarboxylase activity, potential open reading frames (ORFs) were identified and compared to ORFs in the NCBI genomic database by BLASTP as described by Kosuge et al. (2006). No regions of homology were found, indicating that B. breve M-16V does not express genes known to induce biogenic amine formation. Functional analyses evaluating the formation of biogenic amines was indirectly determined in vitro by analyzing the pH of a solution containing amino acid substrates before and after the addition of the B. breve M-16V as described in Appendix 4. Compared to Lactobacillus buchneri L586 and Enterococcus faecalis E213, which form histamine and tyramine, respectively, there was no appreciable increase in pH when B. breve M-16V was added to histidine- and tyrosine-containing solutions. These results indicate that B. breve M-16V does not form biogenic amines".*

10.3.3.3 Ammonia production

According to the GRAS *"Ammonia production by B. breve M-16V was determined in vitro using a colorimetric assay as described in Appendix 4. Compared to medium harvested from E. faecium ATCC 19434 and L. rhamnosus LW744, two types of bacteria known to produce and not produce ammonia, respectively, ammonia was undetectable in the medium harvested from B. breve M-16V cultures".*

10.3.3.4 Bile salt deconjugation

According to the GRAS *"Bile acids may prevent bacterial overgrowth in the small intestine (From GRAS Notice 000455: Bifidobacterium breve M-16V: Lorenzo-Zuniga et al., 2003; Ding et al., 1993) and it is generally believed that probiotic bacteria are able to survive their passage through the gastrointestinal tract. Bile salt hydrolase activity is common to all bifidobacteria, including B. breve (Tanaka et al., 1999). In vitro analyses showed that B. breve M-16V hydrolyzed the conjugated bile acids taurocholic and glycocholic acid to the primary bile acid cholic acid and hydrolyzed glycochenodeoxycholic and taurochendeoxycholic acid to chenodeoxycholic acid (Table 11).*

The analyses also showed the B. breve M-16V did not dehydroxylate cholic acid and chenodeoxycholic acid to the secondary bile acids deoxycholic and lithocholic acids (Table 11). The methods used to determine B. breve M-16V bile salt deconjugation and dehydroxylation are described in Appendix 4".

10.3.3.5 Azoreductase or nitro reductase activity

According to the GRAS *"Previous studies have shown that B. breve does not have azoreductase or nitroreductase activity (Nakamura et al., 2002). To determine whether the genome of B. breve M-16V contains genes capable of conferring azoreductase and nitroreductase activity, potential ORFs were identified and compared to sequences in the NCBI genomic database (nr, 20120604) using BLASTP as described by Kosuge et al. (2006). B. breve M-16V contained no regions with homology to other genes known to confer azoreductase activity. However, three potential ORFs had significant homology (E-values less than 1 x 10 -50) to nitroreductases expressed by other types of bifidobacteria and two of the three potential ORFs, g1083 and g1373, had less than 50% homology to nitroreductase genes expressed by other types of bacteria (Appendix 9). To confirm that B. breve M-16V did not spontaneously acquire azoreductase activity, the bacterium was grown on agar plates containing Direct Blue 15, Sunset Yellow FCF, and Amaranth food dye E213, and the plates were analyzed for discoloration as described in Appendix 4. Compared to C. perfringens ATCC 13124 and Lactobacillus fermentum L421, which reduce and do not reduce azo dyes, respectively. No discoloration was observed for B. breve M-16V".*

10.3.4 Hemolytic activity

According to the GRAS *"The hemolytic potential of B. breve M-16V was assessed by identifying potential ORFs and compared to the NCBI genomic database (nr, 20120604) as described by Kosuge et al. (2006). BLASTP analyses revealed that the genome of B. breve M-16V contains one gene, g0647, having homology to other bifidobacterial gene products that may be involved in hemolysis (Appendix 10). Hemolytic activity was evaluated by plating and culturing B. breve M-16V on agar plates containing sheep blood for 72 hr as described in Appendix 4. In contrast to Listeria ivanovii subsp. ivanovii ATCC 19119, which is known to cause red blood cell lysis, hemolysis was undetectable for B. breve M-16V and B. longum subsp. longum BB536, a strain of bifidobacteria that has already been GRAS (GRN 268) (Figure 11). A study conducted by Sanquin Research also found that B. breve M-16V had no deleterious effects on erythrocytes (Appendix 11, B. breve M-16V referred to B602)"*.

10.3.5 Effects on platelet aggregation or viability

According to the GRAS "*The potential for B. breve M-16V to induce platelet aggregation and cell death, as measured by cell surface expression of phosphatidylserine was evaluated by Sanquin Research (Appendix 12; B. breve M-16V is referred to as B. breve B602). B. breve M-16V did not induce platelet aggregation nor did it induce cell death. Furthermore, BLASTP analyses showed that the B. breve M-16V genome does not contain any regions with homology to genes expressed by other bacteria known to cause platelet aggregation*".

10.3.6 Adherence to mucus and/or human epithelial cells and cell lines

According to the GRAS *"The mucin layer of the gastrointestinal tract helps protect underlying epithelial cells from digestive enzymes present in gastric juice, shear generated by digestive processes, and ingested pathogens (Johansson et al., 2011). Thus, any alteration in the mucus layer may compromise the host. B. breve M-16V was unable to grow in medium or on agar containing mucin and does not induce mucin degradation in vitro (Abe et al., 2010)"*.

10.3.7 Toxin production

Based on in vivo safety in animal studies and according to GRAS *"to assess the toxic and pathogenic potential of B. breve M-16V, predicted amino acid sequences, and, in some case, mRNA sequences of genes expressed by B. breve M-16V were compared to those expressed by toxic and pathogenic bacteria using BLASTP. Although B. breve M-16V expressed a variety of potential gene products that are highly homologous (Evalues less than 1x10-5°) to those gene products expressed by Arcanobacterium pyogenes, Pseudomonas aeruginosa, Clostridium perfringens strain 13, and Staphylococcus aureus N315 (Appendix 8A), subsequent BLASTP analyses also showed that these potential gene products are also highly homologous (E-values less than 1x10 -41) to gene products expressed by other species of Bifidobacterium including B. infantis, B. longum, B. catenulatum, B. adolescentis, and B. bifidum (Appendix 8B). Importantly, BLASTP analyses only identify regions of homology and do not evaluate the expression and functionality of the homologous regions. Furthermore, the toxicity and/or pathogenicity of bacteria is dependent on the coordination of environmental stimuli and the expression and activation of a variety of other gene products (Wassenaar and Gaastra, 2001). Thus, the regions of homology to A. pyogenes, P. aeruginosa, C. perfringens strain 13, and S. aureus N315 may not confer toxicity or pathogenicity. Studies evaluating the toxicity and pathogenicity of B. breve M-16V are included in section V.B.2 and indicate that B. breve M-16V behaves in a fashion similar to B. longum BB536, which is GRAS for use in conventional foods (GRN 268)".*

10.3.8 Acute, subacute, and chronic animal toxicity

According to the GRAS *"Acute, subacute, and chronic animal toxicity after oral administration of the B. breve M16-V was evaluated in two publications: Abe et all., (2009), and a report TNO toxicology and pharmacology.*

The report by TNO toxicology and applied pharmacology (2005) describes a 28-day toxicity trial with daily exposure to B. breve M-16V in rats. An increase in the number of blood monocytes was observed in female rats, while in male rats the weight of the thymus and spleen increased slightly. These observations were considered of no toxicologic relevance, and no other abnormalities associated with treatment were observed. Abe et al. (2009) conducted a series of studies evaluating the toxicity of B. breve M-16V in rats. In a single dose challenge, the investigators did not report any histopathological findings attributable to treatment upon necropsy after 14 days. In a 90-day repeated dose trial. The animals were monitored with respect to bodyweight and feed consumption. Urine and blood were analyzed extensively. Finally, a large number of histological examinations were carried out at the end of the study period. The only abnormality observed was an increased Major Histocompatibility Complex (MHC) value in the group of male rats treated with B. breve. No adverse treatment effects were observed. Tissue translocation potential to determine whether B. breve M-16V could translocate the epithelial lining of the gastrointestinal tract, mice were fed B. breve M-16V for five days, treated with the immunosuppressive agent cyclophosphamide 4, 6, and 8 days after beginning bacterial treatment, and euthanized four days later (Appendix 4). Blood and liver, spleen, and mesenteric lymph node homogenates were cultured on BL agar under anaerobic conditions and the resulting colonies were counted. B. breve M-16V and B. longum subsp. longum BB536 were not detected in the blood, liver, spleen, and mesenteric lymph nodes. These results indicate that B. breve M-16V did not translocate outside the gastrointestinal tract, even in immunocompromised hosts.

Intravenous challenge Bacterial-induced toxicity was determined by intravenously administering increasing amounts of B. breve M-16V or B. longum BB536 to healthy and immunocompromised mice

and monitoring the mortality rate over 14 days (Appendix 4). Toxicity of the opportunistic human pathogen P. aeruginosa was used as a positive control and induced death in heathy mice at approximately 106 cfu. In contrast, B. breve M-16V caused deaths in a dose-dependent fashion at doses greater than 10 ^{9} cfu, which was similar to that induced by B. longum BB536. In immunocompromised mice, B. breve M-16V caused deaths at doses greater than 0.3 x 10^{9} cfu, which was like what was observed in healthy mice and was approximately 10-fold less than that induced by B. longum BB536. These results show that B. breve M-16V is not highly toxic or pathogenic".

10.4 Appendix: OHAT scores for primary studies on infant formula

Table 10.4-1. An overview of the RoB rating and the classification into tiers for adverse events (AE) for each RCT study.

	Key RoB criteria			Other RoB criterion					
Author, year	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	Tier
Abrahamse-Berkeveld et al., 2016	+	+	++	+	+	-	+	-	**1**
Burks et al., 2015	+	+	+	+	-	+	+	-	**1**
Candy et al., 2018 (ASSIGN study)	+	++	+	++	+	+	+	-	**1**
Chatchatee et al., 2022	++	++	+	+	++	+	+	-	**1**
Chua et al., 2017	++	++	-(NR)	+	++	+	-	-	**2**
Fox et al., 2019 (ASSIGN study)	+	+	+	++	+	+	+	-	**1**
Harvey et al., 2014 (study 1)	+	+	+	+	-	-	+	-	**2**
Harvey et al., 2014 (study 2)	-	+	-(NR)	+	-	-	-	-	**3**
Phavichitr et al., 2021	-	+	-(NR)	+	-	+	-	-	**3**
van der Aa et al., 2010	++	++	+	++	+	++	+	-	**1**
Wang et al., 2021	+	+	+	++	++	++	++	-	**1**

Table 10.4-2. An overview of the RoB rating and the classification into tiers for infant growth indicators for each RCT study.

	Key RoB criteria			Other RoB criterion					
Author, year	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Abrahamse-Berkeveld et al., 2016	+	+	++	+	+	-	+	-	**1**
Burks et al., 2015	+	+	+	+	-	+	+	-	**1**
Candy et al., 2018 (ASSIGN study)	+	++	+	++	+	+	+	-	**1**
Chatchatee et al., 2022	++	++	+	+	++	+	+	-	**1**
Chua et al., 2017	++	++	-(NR)	+	++	+	-	-	**2**
Fox et al., 2019 (ASSIGN study)	+	+	+	++	+	+	+	-	**1**
Harvey et al., 2014 (study 1)	+	+	+	+	-	-	+	-	**2**
Phavichitr et al., 2021	-	+	-(NR)	+	-	+	-	-	**3**
van der Aa et al., 2010	++	++	-(NR)	++	+	++	+	-	**2**
Wang et al., 2021	+	+	++	++	++	++	++	-	**1**

Table 10.4-3. An overview of the RoB rating and the classification into tiers for gastrointestinal symptoms for each RCT study.

	Key RoB criteria			Other RoB criterion					
Reference	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Abrahamse-Berkeveld et al., 2016	+	+	+	+	+	-	+	-	**1**
Burks et al., 2015	+	+	+	+	-	+	+	-	**1**
Candy et al., 2018	+	++	+	++	+	+	+	-	**1**
Chua et al., 2017	++	++	-	+	++	+	-	-	**2**
Fox et al., 2019	+	+	+	++	+	+	+	-	**1**
Harvey et al., 2014 (study 1)	+	+	+	+	-	-	+	-	**2**
van der Aa et al., 2010	++	++	+	++	+	++	+	-	**1**
Wang et al., 2021	+	+	+	++	++	++	++	-	**1**

For Sorensen et al., 2021a (observational design), the RoB rating for gastrointestinal symptoms is given in Table 10.4-6.

Table 10.4-4. An overview of the RoB rating and the classification into tiers for skin symptoms including SCORAD for each RCT study

	Key RoB criteria			Other RoB criterion					
Reference	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Abrahamse-Berkeveld et al., 2016	+	+	+	+	+	-	+	-	**1**
Burks et al., 2015	+	+	+	+	-	+	+	-	**1**
Candy et al., 2018	+	++	++	++	+	+	+	-	**1**
Chatchatee et al., 2022	++	++	++	+	++	+	+	-	**1**
Fox et al., 2019	+	+	++	++	+	+	+	-	**1**
Hulshof et al., 2018	-(NR)	++	++	+	-	+	++	-	**2**
Taniuchi et al., 2005	- -	-	+	-	-	-	+	-	**3**
van der Aa et al., 2010	++	++	++	++	+	++	+	-	**1**

For Sorensen et al., 2021a (observational design), the RoB rating for skin symptoms is given in Table 10.4-6

Table 10.4-5. An overview of the RoB rating and the classification into tiers for airway symptoms for each RCT study.

	Key RoB criteria			Other RoB criterion					
Reference	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Abrahamse-Berkeveld, 2016	+	+	+	+	+	-	+	-	**1**
Burks, 2015	+	+	+	+	-	+	+	-	**1**
Candy, 2018	+	++	+	++	+	+	+	-	**1**
Fox, 2019	+	+	+	++	+	+	+	-	**1**
van der Aa, 2011	+	++	+	++	+	+	++	-	**1**

For Sorensen et al., 2021a (observational design), the RoB rating for airway symptoms is given in Table 10.4-6

Table 10.4-6. An overview of the RoB rating and the classification into tiers for gastrointestinal (GI)-, skin-and airway symptoms in one observational study (Sorensen et al., 2021a)

	Key RoB criteria			Other RoB criterion				
Reference	4. Did the study design or analysis account for important confounding and modifying variables?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	3. Did selection of study participants result in appropriate comparison groups?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
GI symptoms	+	-	+	++	+	+	-	**2**
Skin symptoms	+	-	+	++	+	+	-	**2**
Respiratory symptoms	+	-	+	++	+	+	-	**2**

10.5 Appendix: OHAT score for primary studies on enteral feeding published after 2018

Table 10.5-1. An overview of the RoB rating and the classification into tiers for one RCT study.

		Key RoB criteria			Other RoB criterion					
Reference	**Outcome**	6. Were the research personnel and human subjects blinded to the study group during the study?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	1. Was administered dose or exposure level adequately randomized?	2. Was allocation to study groups adequately concealed?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Agrawal et al., 2020	Primary outcome, MSEL	+	+	+	++	++	- -	+	-	**1**
	Secondary outcome: Developmental, Dimensional and Diagnostic Interview (3Di)	- (NA)	+	+	++	++	- -	+	-	**2**
	Tertiary outcome: Developmental NEuroPSYchological assessment–2nd Edition (NEPSYII)	+	+	+	++	++	- -	+	-	**1**
	Tertiary outcome: Children's Communication Checklist–2nd edition (CCC-2)	- (NA)	+	+	++	++	- -	+	-	**2**
	Tertiary outcome: Social Responsiveness Scale (SRS)	- (NA)	+	+	++	++	- -	+	-	**2**
	Tertiary outcome: Vineland Adaptive Behavioural Scales–2nd edition (VABS-II)	- (NA)	+	+	++	++	- -	+	-	**2**

Table 10.5-2. An overview of the RoB rating and the classification into tiers for three observational studies.

		Key RoB criteria			Other RoB criterion				
Reference	**Outcome**[1]	4. Did the study design or analysis account for important confounding and modifying variables?	8. Can we be confident in the exposure characterisation?	9. Can we be confident in the outcome assessment?	3. Did selection of study participants result in appropriate comparison groups?	7. Were outcome data complete without attrition or exclusion from analysis?	10. Were all measured outcomes reported?	11. Were there no other potential threats to internal validity?	**Tier**
Athalye-Jape et al., 2020	TFF[1]	+	+	-	- -	+	+	+	**3**
	NEC[2]	+		-					**3**
	LOS[3]	+		-					**3**
	Weight	-		-					**3**
	Length	-		-					**3**
Inage et al., 2021	Hyperglycemia	+	-	+	+	+	+	- -	**2**
Priyadarshi et al., 2021	Probiotic sepsis, *B. Breve* M-16V	+	+	+	**(n/a[4])**	+	+	+	**1**

[1]Time to full feeds.
[2]Necrotising enterocolitis.
[3]Late-onset sepsis.

[4]n/a, not applicable. Comparison group not mandatory for occurrence of strain-specific probiotic sepsis .

10.6 Appendix: Customization of OHAT criteria

Protocol for questions 8, 9 and 11 in the OHAT risk of bias assessment for formula studies.

8. Can we be confident in the exposure characterization?

Characteristics that must be known to have confidence in the exposure characterization:

1. Known strain/known manufacturer
2. Known concentration of breve
3. Known control (formula with or without prebiotics, relevant for GI-symptoms which may occur also with control formula)
4. Known total volume of formula intake (relevant for compliance assessment, most relevant for weight and GI-symptoms)
5. Described compliance, and similar in test and control (in light of intervention; target volume, exclusive formula feeding)

Explanation: Difference between direct and indirect evidence. If direct evidence of differences between groups: -- (definitely high)

Example: Known control, but differences in content between test and control.

Template **OHAT 8) Can we be confident in the exposure characterization?**	**++** **Definitely low**	**+** **Probably low**	**- (NR)** **Probably high**	**--** **Definitely high**
Number of criteria that needs to be reported. "Reported" is placed in field regarded as mandatory	5/5	4/5	3/5	<2/5
1 Known strain/known manufacturer	Reported	Reported	Reported	Reported
2 Known concentration of *B. breve* M-16V	Reported	Reported		
3 Known control	Reported	Reported	Reported	
4 Known total volume of formula intake	Reported			
5 Described compliance, and similar in test/control	Reported/mandatory	Reported/mandatory		

Examples **OHAT 8) Can we be confident in the exposure characterisation?**	**++** **Definitely low**	**+** **Probably low**	**- (NR)** **Probably high**	**--** **Definitely high**
Reference	Abrahamse-Berkeveld et al., 2016	Wang et al., 2021		
Number of criteria described/reported	5/5	4/5	3/5	<2/5
1 Known strain/known manufacturer	Probiotic strain *B. breve* M-16V (1·3 × 10exp9 colony-forming units per 100 ml formula; Morinaga Milk Industry Co., Ltd).	*B. breve* M-16V (3 x 10exp7 CFU/g).	Reported	Reported
2 Known concentration of breve	*B. breve* M-16V (1·3 × 10exp9 colony-forming units per 100 ml formula;	*B. breve* M-16V (3 x 10exp7 CFU/g).		
3 Known control	Standard extensively hydrolysed (63 % of proteins <1000 Da) whey protein-based powder products with established anti-allergenic properties and intended to provide adequate nutritional support of infants in the first 6 months of life (20,21). The formulas were isoenergetic and contained per 100 ml a similar amount of 66 kcal (276 kJ) energy, 1·6 g protein, 6·8 g carbohydrate, 3·6 g lipid, vitamins and minerals.	The control was a commercially available standard infant formula (IF), Aptamil Pronutra, and is an intact cow's milk protein-based IF containing a prebiotic mixture of 0.8 g/100 mL scGOS/lcFOS (9:1 ratio). Although energy content was very similar in both products, it should be noted that the test formula contained a higher concentration of carbohydrates (7.2 versus 6.7 g/100 mL) compensated by a lower level of fat content (3.4 versus 3.5 g/100 mL) compared with the control IF	Reported	
4 Known total volume of formula intake	Average daily intake of the formula (ml/d) was calculated based on the parents' monthly records. AND Table 4. Daily formula intake of the synbiotic and control groups in the per-protocol population*	The average daily IF intake was similar among IF-fed groups over the intervention period (Table 2). The mean (SD) daily IF intake at V2 was 809 (215) mL/d and 803 (176) mL/d for the test and control groups, respectively, increasing to 961 (239) mL/d in the test group and 975 (194) mL/d in the control group at V5 (PP). AND Table 2 Daily parent-reported infant formula intake per day and per kilogram body weight in the per-protocol population*		
5 Described compliance, and similar in test/control	Infant were regarded compliant if: 1) they had an average daily intake of >400 ml; (2) the average daily intake was higher than their age- and sex-specified minimum intake	Indirectly reported by flow chart in Figure 1		

	requirements; (3) the left-over, defined as prepared minus consumed, was below 25 %.			

9. Can we be confident in the outcome assessment?

Outcome categories based on papers	Methods	++ Definitely low	+ Probably low	- (NR) Probably high	-- Definitely high
Adverse events (AE)	Active monitoring by clinician vs parental report, prospective vs retrospective reporting, definitions of e.g. "serious", use of coding system for AE, how was intervention (not) attributed to *B. Breve*?	Clear description of monitoring method, review of events by clinician, use of coding system or definition of "serious" or "not serious", process for (not) attributing adverse event to exposure/intervention	Monitoring by parental report/diary with prospective reporting. Fewer criteria than for ++	Parental report with long recall period OR no methods description	Unblinded intervention (open study). Different follow-up/methods for intervention and controls.
Infant growth/ anthropometrics	Clinical examination or parental report,	Indicators specified, calibrated weight, precision given, multiple measurements, see Wang 2021 (benchmark)	Indicators specified, Measured in a clinical setting without further details	Parental report of child's growth	Parental report in unblinded intervention/open study. Different follow-up/methods for intervention and controls.
Symptoms reporting: gastrointestinal (GI) tolerance, skin, airways	Clinical examination vs parental report, prospective vs retrospective registration, established/validated instrument or not	Clinical examination, standardized and validated instrument for symptom score, prospective registration or short-term recall	Parental reported diary, prospective	Parental report with long recall period OR no methods description	Unblinded intervention, open study. Different follow-up/methods for intervention and controls.
Biomarkers blood or plasma	Timepoint/repeats, treatment of samples, lab/analysis method, reference range/normal range	Timepoint/repeats and method described. Reference ranges/cut-offs given	Method described	No methods description	Differences in samples or analysis of intervention and controls

11. Were there no other potential threats to internal validity?

Affiliation with industry by authorship: - (probably high)

Co-exposure such as complementary feeding or medication that can affect health outcomes (antibiotics use or topical steroids): - (probably high)

10.7 Results formula feeding: biomarkers of safety

Table 10.7-1. Results from studies on infant formula that report on blood or plasma biomarkers reflecting safety.

Author, year	Biomarkers measures	Results reported	VKM note
Burks et al., 2015	Plasma albumin, plasma pre-albumin, ferritin, total iron binding capacity, haemoglobin, haematocrit, blood urea nitrogen, potassium, calcium, alkaline phosphatase, white blood cells, red blood cells (RBC), mean cell volume, platelets, sodium, creatinine, chloride	Significant differences found between the study groups regarding haemoglobin, haematocrit, RBC, and alkaline phosphatase. However, these and all other values were within reference ranges.	Reference ranges not reported, data not shown.
Harvey et al., 2014 (study 1)	Blood urea nitrogen, creatinine, sodium, potassium, chloride, and carbon dioxide	Laboratory parameters were within the specified normal ranges and were not statistically different between the study groups.	Reference ranges not reported, data not shown.
Abrahamse-Berkeveld et al., 2016	Unspecified plasma makers of renal and liver function	No differences in any of the other blood parameters were observed between the study groups.	Reference ranges not reported, data not shown.
van der Aa et al., 2010	Unspecified renal and liver function based on blood sample	Renal and liver function did not differ between the two groups.	Reference ranges not reported, data not shown.

10.8 Examples of exposure calculations

Three different examples have been included to illustrate the exposure calculations (Examples 1-3, shown below). The examples include 1) formula as full diet for the 97th weight-for-age-percentile, 2) enriched formula scenario for the 50th weight-for-age percentile, and 3) formula as supplementary diet for the 97th weight-for-age-percentile. The values are shown for boys aged 12 months only as this group is expected to have the highest exposure to *B. Breve* M-16V due to the highest body weight and energy requirement.

Table 10.8-1. Example 1. Exposure calculation for formula as full diet for boys aged 12 months in 97th weight-for-age percentile.

Age (months)	Energy requirement (kcal/kg/day)	Weight 97th percentile (kg)	Energy (kcal/day)	Formula (dl/day)	Dose of *B. breve* M16-V (10^9 cfu day)
12	81	11.8	956	14.5	2.13

Table 10.8-2 Example 2. Exposure calculation for enriched formula as full diet for boys aged 12 months in 50th weight-for-age percentile.

Age (months)	Energy requirement (kcal/kg/day)	Weight 3rd percentile (kg)	Energy (kcal/day	Formula (dl/day	Dose of *B. breve* M16-V (10^9 cfu/day)	
Enrichment					0.5 scoop extra	1 scoop extra
12	81	7.8	632	9.6	2.00	2.27

Table 10.8-3. Example 3. Exposure calculation for formula as supplementary diet for boys on 97th weight-for-age percentile.

Age (months)	Formula volume (mL/kg/day)	Weight 97th percentile (kg)	Formula (dl/day)	Dose of *B. breve* M16-V (10^9 cfu /day)
12	61	11.8	7.2	1.06

10.9 List of supplementary files

The following supplementary files are available with the report at VKM.no

- Study protocol
- Screened studies and reasons for exclusion (Excel)
- ROBIS scoring sheets for review studies
- Exposure calculations (Excel)