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Research Digest

Tony Gentilcore ◆ 5 Year Anniversary Edition

From the Editor

First, we want to thank you for taking the time to check out the Examine.com Research Digest (ERD). We feel a connection to those who love to get their hands dirty, wading through interesting and complex topics in nutrition and supplementation.

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As our reputation grew, health professionals started asking if they could get continuing education credits from reading our reviews. We responded with ERD, which covers new research in depth, using editors and reviewers from academic fields ranging from neuroscience to immunology. Each month, ERD looks at eight recent papers that are both interesting and practical, and presents them in an easy-to-read and graphically pleasing manner. We are now approved for CECs from NSCA, NASM, The Academy, and more.

Tony has always been a big supporter of ERD, so we made this special anniversary issue for his readers, containing five ERD articles he thought you would find interesting.

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"There's a lot of shadiness out in the diet/supplement world, and Examine.com serves as the shadiness police."
- Tony Gentilcore

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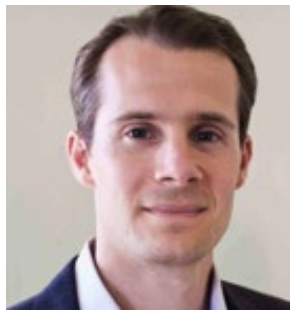

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A shot to the gut

Acute Binge Drinking Increases Serum Endotoxin and Bacterial DNA Levels in Healthy Individuals

Introduction

Half the alcohol consumed in the United States is consumed during a binge, which is defined as having five or more drinks in a row. About one in six Americans binge drink each month. Binge drinking is a risk factor for a host of problems (with some shown in Figure 1), from behavioral issues like engaging in more risky sex and an increase in violence to health issues such as high blood pressure, heart attacks, and liver disease. Binge drinking's definitely a problem - but one that remains incompletely understood. One relatively unexplored area is the role of bacteria in the negative outcomes associated with drinking.

Animal and human studies have recently uncovered that a component of the outer membrane of certain kinds of bacteria (Gram-negative ones, specifically) called lipopolysaccharide (LPS) is often found in higher levels in the blood of people with some alcoholic and nonalcoholic liver diseases. LPS is sometimes called endotoxin, since it's a toxic part "inside" of the bacterial membrane that causes a pretty big immune response when released. Furthermore, antibiotics have been shown to mitigate liver damage in rats exposed to alcohol over longer time periods. From this evidence, it's been hypothesized that increased LPS and other bacterial components that enter the blood after drinking may lead to an immune response, which in turn can contribute to liver damage.

While many of the early studies looking at a possible role for bacteria in alcohol-induced liver disease examined long-term alcohol exposure, more recent experiments have found that bingeing can also lead to increased LPS levels, accompanied by molecular markers of inflammation.

Some questions remain, however. It's not completely clear if this effect can occur in healthy humans after just one binge drinking session. Also, while it's well-known that alcohol leads to more LPS entering the bloodstream, is there any evidence that bacteria themselves or other bacterial components enter the blood acutely? That's what this study intended to explore.

A bacterial component called LPS tends to be found in higher levels in the blood of heavy drinkers. The immune response to it may contribute to alcohol-induced liver disease. This study explored whether LPS and bacteria can enter the blood of healthy individuals after one binge drinking session.

Figure 1: Some problems associated with binge drinking

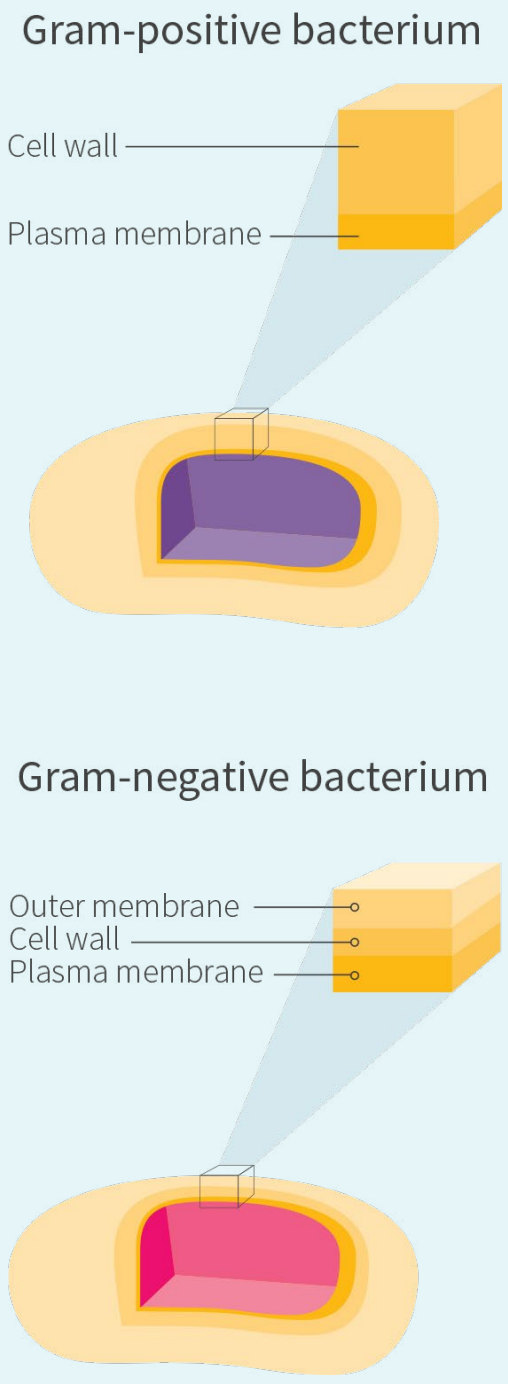
References: 10th Special Report to the US Congress on Alcohol and Health. NIAAA. 2000 Jun.
Wechsler H, et al. JAMA. 1994 Dec.

Gram-positive and Gram-negative bacteria

In the late 19th century, the Danish scientist Hans Christian Gram was attempting to find ways of seeing bacteria more easily under the microscope by staining them with dye. He discovered that some bacteria took up his dye very easily, which made them very visible under the microscope. However, many bacteria didn't. The bacteria that dyed easily are known as "Gram-positive" bacteria, and those that didn't are known as "Gram-negative."

This difference is due to the composition of the outside of the bacteria. Gram-positive bacteria have a thick cell wall that stains well, shown in Figure 2. This big cell wall makes Gram-positive bacteria physically tougher than Gram-negative ones, but Gram-negative bacteria have an outer membrane that protects its relatively thinner cell wall which makes them chemically tougher. This outer membrane can make it harder for antibiotics to penetrate, making Gram-negative bacteria harder to kill with drug treatment as a rule of thumb. The outer membrane of Gram-negative bacteria also contains LPS as a component, which causes a large immune response and can lead to septic shock. Since this is part of the structure of the bacterium, it's known as an endotoxin. Gram-positive bacteria, on the other hand, generally secrete their toxins (again, as a rule of thumb), and so these are called exotoxins.

Figure 2: Gram-positive versus Gram-negative bacteria



Who and what was studied?

Healthy people with no previous history of alcohol issues were enrolled in the study. Men who had 12 or more drinks per week and woman who had 9 or more were excluded from the study.

A total of 11 men and 14 women ages 21-56 were given 2mL vodka per kg bodyweight mixed with a mixture of strawberry and orange juice after abstaining from alcohol 48 hours before the study. This is equal to about 4-5 shots of vodka for an overweight person (which the participants were, on average). But keep in mind that the exact dose of alcohol depended on the participant's bodyweight, so some people had more, and some had less. After drinking, blood was drawn every 30 minutes for 4 hours and then once more 24 hours afterwards.

The researchers looked for three different kinds of things in the blood. Firstly, they checked the alcohol level in the blood to make sure that the alcohol was being absorbed and to see how blood levels correlated with the other blood measurements. They also looked at the amount of bacterial DNA present in the blood to see if bacteria (or parts of their DNA at the very least) were entering the bloodstream more easily after drinking. In addition, they also looked at LPS levels as well as some immune components of the blood to see how the immune system was behaving. Two of these components, specifically

lipoprotein binding protein (LBP) and soluble CD14 (sCD14) are "acute phase" proteins that are normally released early on in the inflammatory response.

The same procedure and measurements were done on age- and gender-matched controls who were given an equal volume of orange juice to drink with no alcohol in it.

25 healthy people who had no history of alcohol problems and normally only drank around 1-2 drinks per day tops were recruited for this study. They drank roughly 4-5 shots of vodka mixed with strawberry and orange juice quickly, and then their blood was measured for inflammatory and bacterial markers. These results were compared to those obtained from individuals in the control group who were abstinent from alcohol and who were just given orange juice.

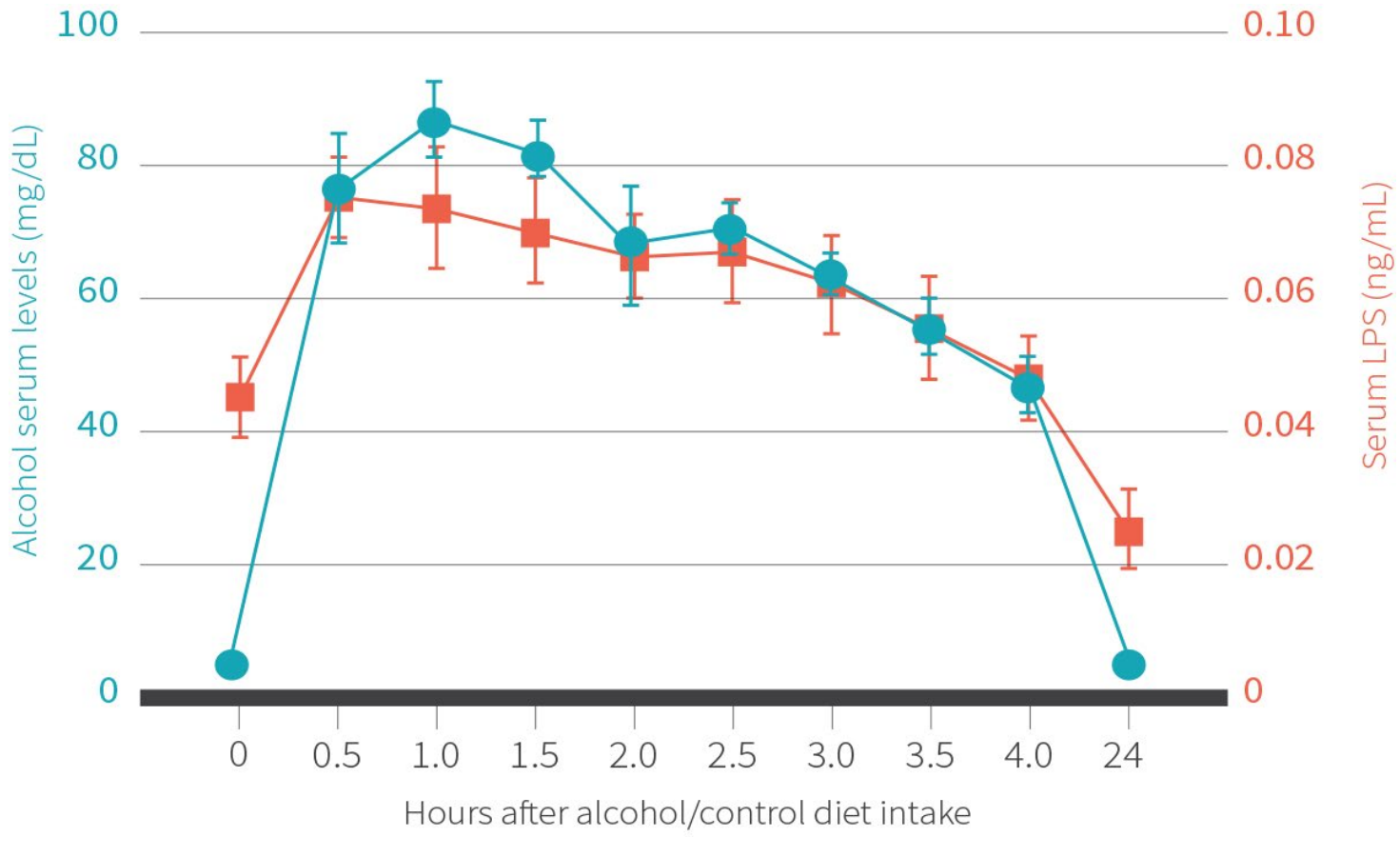
What were the findings?

The researchers confirmed every partygoer's experience ever by finding that consuming alcohol increases the blood alcohol level. The blood alcohol level peaked at about an hour after consuming the beverage, and decreased from there. The decrease was slower in women.

What was more interesting are the results shown in Figure 3: the amount of LPS found in the drinkers' blood tracked the blood alcohol content. The amount of LPS rose sharply 30 minutes after consuming the alcohol, and remained elevated for 3 hours before returning to baseline 24 hours after alcohol ingestion. Women's LPS levels tended to be higher than men's. Acute phase proteins also rose in the drinkers and remained elevated 24 hours after drinking. In comparison, controls just drinking orange juice had minute levels of LPS that were about 20 times lower than the alcohol group's peak, with no change in acute phase proteins.

To make sure that LPS would induce an immune response at the concentrations found in the drinkers' blood, the researchers drew blood from healthy volunteers and added LPS to it at a level similar to that found during the experiment above. Sure enough, an immune response was elicited, as measured by an increase in several markers of inflammation.

Figure 3: Blood alcohol and LPS levels track each other



Bacterial DNA increased over 1.5 times the baseline levels for the alcohol-drinking group, and remained elevated 24 hours afterwards, whereas a constant basal level of bacterial DNA was observed for the control group.

Drinking a high dose of alcohol during just one session seemed to cause an increase in both bacterial DNA and a bacterial toxin known as LPS in the bloodstream, along with an increased immune response.

What does the study really tell us?

Connecting the dots from the last section, this study suggests that an alcohol binge in healthy individuals can lead to components of bacteria entering the bloodstream from the gut as well as to an acute immune response.

We can't say for certain whether intact bacteria are actually invading the bloodstream from the gut, however. The researchers technically only measured the blood for bacterial DNA and one component of Gram-negative bacterial membranes. The gold standard for seeing whether bacteria were entering and thriving in the blood stream would be a blood culture, where one attempts to grow bacteria from the blood on a petri dish. This wasn't done. Bacterial blood infections tend to be somewhat serious, and often cause symptoms that are often noticeable. Yet we don't see people pouring into the ER for a course of antibiotics after a night out on the town. So it's likely that the gut is getting leaky from the alcohol, and that some bacterial parts are being absorbed into the blood. Maybe some bacteria enter as well, but this probably won't often lead to a full-blown bacterial infection of the blood.

Indeed, much research to date seems to indicate that it's bacterial components that enter the body through a variety of mechanisms, and not whole bacteria in large numbers, although the question hasn't been studied too closely. However, the evidence found here is certainly consistent with whole bacteria entering the blood, at least to some degree.

It can't be determined for certain that the bacterial components entering the blood caused the acute immune response in this case. Technically, all that was observed from the blood draws was a correlation between acute phase inflammation markers and the concentration of bacterial components in the bloodstream. It's true that the researchers checked to see that LPS levels similar to those found in the drinkers' blood could induce an immune response. But strangely, the researchers looked at different inflammatory cytokines in this experiment than the ones they measured from the drinkers' blood.

What can be said with more certainty is that some bacterial components do enter the bloodstream after a one-time binge drinking session in healthy individuals, and that this correlates with an acute inflammatory response. However, this study alone can't speak to whether or not this observation is of much significance for one's health in the long run.

While bacterial molecules increased in the blood after binge drinking, this doesn't necessarily imply that binge drinking can lead to bacterial invasion and infection. And while this study showed that binge drinking increased some markers of inflammation, which correlated with the rise in bacterial markers, this doesn't say much about the longer-term health implications.

The big picture

The fact that people have higher LPS in their blood after drinking has been known by observation since at least 1987. However, the mechanisms by which this occurs have only come to light more recently. Alcohol seems to set off a cascade of changes leading to lower production

of tight junction proteins, which help stich intestinal cells together so that little can pass through them. Alcohol also seems to eat away at the gut wall on its own, causing direct physical damage. Finally, chronic alcohol consumption also seems to alter the gut microflora, favoring Gram-negative bacteria which harbor LPS. The more Gram-negative bugs there are, the more their components can enter the bloodstream.

Strangely, increased LPS entering the blood may actually create a positive feedback loop, leading to more alcohol drinking. This effect has been observed in mice administered LPS, which stimulated immune signalling that ultimately altered the reward center of the mice's brains. The hypothesis that immune changes lead to behavioral changes is bolstered by the fact that suppressing immune activity negates increased alcohol consumption in mice. It's unknown whether this effect is also true in humans, although changes in some genes involved in the immune system have been seen in the brains of human alcoholics.

Binge drinking doesn't just affect inflammation. It also injures the liver and contributes to fat accumulation there. Somewhat paradoxically, there's also some evidence that binge drinking harms the immune response to viruses and bacteria overall, even though this study showed an increase in inflammation. Also, the hangover the following day makes it more difficult to retrieve memories. Perhaps surprisingly, though, there's some controversy around exactly how much binge drinking affects cognition; some studies have come up negative and laboratory experiments don't necessarily coincide with more observational studies.

This study fits into the big picture by suggesting another possible mechanism by which binge drinking can cause harm. However, it didn't actually measure harm. Instead, it measured molecular markers which suggest that gut bacteria may be associated with the problems caused by bingeing. Hopefully future studies will take a look at whether this mechanism actually leads to some of the damage caused by binge drinking.

“Alcohol seems to set off a cascade of changes leading to lower production of tight junction proteins, which help stich intestinal cells together so that little can pass through them. Alcohol also seems to eat away at the gut wall on its own, causing direct physical damage.”

Alcohol can erode the lining of the gut and change the gut microbiome to favor Gram-negative bacteria, and can contribute to leakage from the gut to the bloodstream. LPS, a component of Gram-negative bacteria, may actually stimulate the brain to crave more alcohol. In addition to making the gut more leaky and inducing inflammation, binge drinking can also harm the liver, the immune system, and cognition.

Frequently asked questions

After a night of binge drinking, how long will it take for my gut to stop leaking?

It's not really known. This study only looked 24 hours out and didn't look at gut leakiness in particular, but instead bacterial products entering the bloodstream. But presuming the bacterial components in the blood are a decent proxy for gut leakiness, then things pretty much return to normal after 24 hours.

Gut leakiness in alcoholics who've stopped drinking has been examined, though, and in that case, it takes up to two weeks for the gut to become less permeable. Another study using different measures of gut permeability actually found that alcoholics didn't have much change in permeability, but did find that acute doses of alcohol increased gut permeability in both the alcoholics and non-alcoholic controls.

Does anything reduce alcohol-induced gut leakage?

Not binging would probably do the trick. But also, one mouse study has suggested that *Lactobacillus rhamnosus* GG helps reduced alcohol-induced intestinal permeability. But this is just one mouse study where they didn't use live culture, but instead components of a culture, so this doesn't speak much to whether probiotic supplementation would work in humans.

What should I know?

This study found that a night of binge drinking led to bacterial parts entering the bloodstream and caused an immune response. It is possible that this process may factor into some of the damage done by drinking heavily. This study didn't measure the long-term effects of this process, so it can't be said for sure whether this process really affects health, though. But these results do suggest a road for future study. ◆

Binging on informed discussion doesn't cause your gut to leak (as far as we know - we couldn't find any studies on this topic), so come on over to the private ERD forum on Facebook to talk about this article!

> "This study fits into the big picture by suggesting another possible mechanism by which binge drinking can cause harm. However, it didn't actually measure harm."

Baby probiotics for prevention of ADHD and Asperger's

A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial

Introduction

Diagnoses of developmental disabilities in children are on the rise in the United States, with increases in attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders such as Asperger's syndrome (AS) leading the trend. Knowing how to stem this tide would be useful. But how?

One tantalizing possibility may be probiotics. Research into the "gut-brain axis" is a hot topic right now, one we've most recently explored in ERD #6 in the article "Can fiber change your emotions?" While the research is still young, mounting animal and human evidence suggests that the gut microbiome can affect brain activity. Since developmental disorders such as ADHD and AS involve the brain, perhaps probiotic supplementation may affect these disorders as well. This idea is bolstered by the fact that many children on the autistic spectrum have concurrent gastrointestinal issues and abnormal gut bacteria. Furthermore, one hypothesis for how ADHD develops, at least in some cases, has to do with immunologic hypersensitivity to environmental triggers. And some of the authors of the study under review found that probiotics are able to mitigate some aspects of this type of hypersensitivity in children.

The sum of these ideas suggest that probiotics in early life could influence the development of ADHD and AS later in life. This study intended to put this theory to the test.

Mounting evidence suggests that gut bacteria may influence brain activity. This presents the possibility that probiotic supplementation could also influence childhood developmental disorders such as ADHD and Asperger's syndrome.

Who and what was studied?

The purpose of this study was to determine whether probiotic supplementation during infancy affected the development of either ADHD or AS by age 13. However, this study is actually a repurposed follow-up of a previous study that looked at whether probiotic supplementation could prevent allergic hypersensitivity in infants. Because of this, the infants recruited for the study all had at least one relative with an allergic disease in order to increase the chances that the infants would also have an allergic disease.

In the original study, 159 mothers were randomized to receive either 10 billion colony-forming units daily of *Lactobacillus rhamnosus* GG (LrGG) in capsule form or placebo four weeks before the infants were born. This commonly studied probiotic is described in Figure 1. After birth, the mothers continued taking the dose if they breastfed their infants. If the infants were not breastfed, they were given the contents of the capsule mixed with water. Most mothers gave the capsules directly to their infants, but some infants never received the capsules. Instead, the breastfeeding mothers took the capsules for the duration of the study. The dosing of the mothers or infants continued for six months after birth.

Stool samples were taken from the infants multiple times, from three weeks of age to 24 months. These samples were used to measure the composition of the infants' gut microbiome using two different methods: fluorescein *in situ* hybridization, which uses fluorescent DNA probes that bind

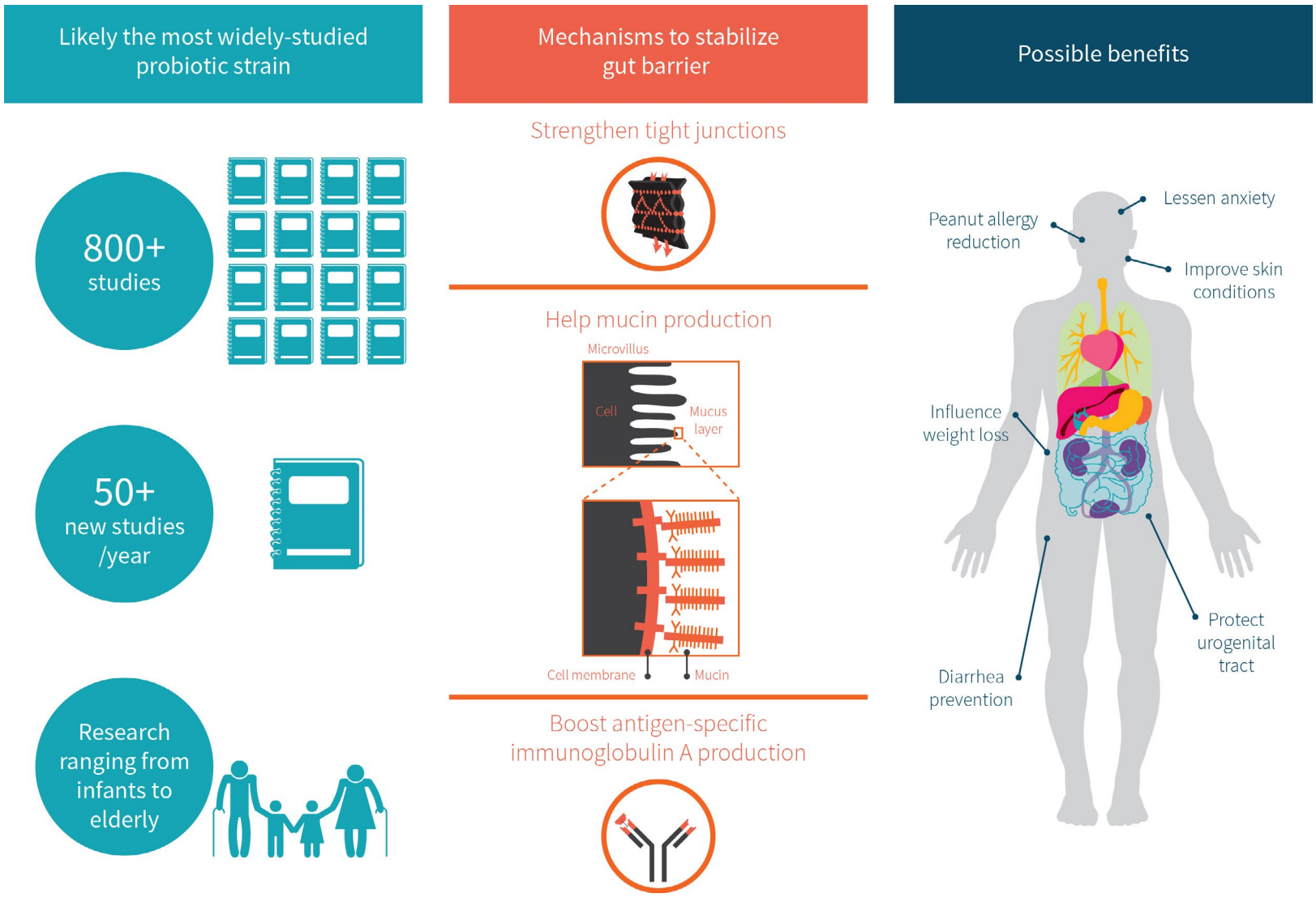
Are ADHD and AS connected?

ADHD involves inattention, impulsivity, and hyperactivity, whereas AS is characterized by stereotyped behavior and impaired social and communication skills. There seems to be little in common between these two disorders at first glance, so why did the authors of this study decide to examine the effect of probiotics on these two seemingly disparate conditions?

One feature of ADHD is that it's rarely seen on its own. In the words of two researchers: "It is the exception, not the rule, to encounter cases with 'pure' ADHD." The same also seems to hold true for AS; attention issues and full-blown ADHD can be found in children with AS as well. The reason why aspects of these conditions may overlap may come down to where they occur in the brain. Both involve some of the same regions of the brain, in what is known as the frontostriatal system. Disorders that arise from this region are thus known as frontostriatal disorders, which include ADHD and AS. Recent neuropsychological evidence suggests that ADHD and AS share some similar brain circuits and both involve problems with managing cognitive processes and emotions, self-control, and executing complex tasks (collectively known as executive dysfunction), implying that both disorders may have some underlying similarities.

So while it seems a bit strange to think that a single intervention could affect the development of two seemingly different disorders such as ADHD and AS, there are some good reasons to put them in one basket for the purposes of this study.

Figure 1: The illustrious Lactobacillus rhamnosus GG



to known sequences of DNA (in this case, specific to microbial species) to identify and count them, and quantitative PCR, which amplifies known segments of the DNA of different microbial species to detect and count them.

In this study, the investigators followed up with the children when they were 13 years old to see if LrGG dosing affected the development of either ADHD or AS. A fecal sample was taken again to analyze the gut microbiome. The children were diagnosed with ADHD or AS using criteria from the International Classification of Diseases (ICD-10) by an experienced psychologist, who was blinded to the treatment groups. In the United States, the Diagnostic and Statistical Manual of Mental Disorders (DSM) is more often used for diagnosis of these conditions. AS was removed from the DSM in 2012 and incorporated into "autism spectrum disorder". Possibly because the current study was conducted outside of the US, ICD was used instead of DSM for diagnosis.

Children (or their mothers if and when the child was breastfed) were randomized to *Lactobacillus rhamnosus* GG supplementation or placebo for up to six months of age. The infants' gut microbiome was analyzed. After the children reached 13 years of age, their gut microbiome composition was again analyzed. The authors examined whether or not supplementation in early life affected diagnosis with ADHD or Asperger syndrome later on.

What were the findings?

Out of the 159 people who participated in the original study, only 75 people participated in the 13-year follow-up. The participation rate was roughly equal between both groups: 40 children (53.3%) of those who received LrGG and 35 (46.7%) who received placebo participated. The two groups had similar characteristics. Those who dropped out for the 13-year follow-up also had similar characteristics to those who remained in the study, except for one notable difference:

Why did the researchers use *Lactobacillus rhamnosus* GG?

Out of all of the different bacteria they could have chosen, why would the authors choose to use LrGG as a probiotic supplement?

The study under review is a follow-up to a previous study whose goal was to examine the prevention of allergic hypersensitivity in infants. The main reason the authors used LrGG in the original study was that they previously found that this strain reversed food allergy-induced gut permeability in rats, and also modulated the immune response in a way that reduced allergic reactions.

The reason why the authors chose this bacterium had little to do with the gut-brain axis, and more to do with the bug's ability to modulate allergic responses. As luck would have it, however, later animal studies suggested that *L. rhamnosus* supplementation could affect brain functioning as well. Perhaps studies like these are what inspired the authors to follow up on their original allergy study and test for psychological effects over a decade after their original allergy study ended.

Science is full of interesting accidents.

the dropouts had significantly fewer exclusively breast-fed months than participants who remained in the study (three months for those who remained in the study, two months for the dropouts).

In terms of psychiatric conditions, three children were diagnosed with ADHD, one child was diagnosed with AS, and two children had both ADHD and AS by age 13. *All* of the diagnosed children were in the placebo group, and all were male. There were no ADHD or AS diagnoses in the LrGG-treated group. These differences in ADHD and AS incidence between the LrGG-treated group and the placebo group were statistically significant.

There were significantly fewer cases of ADHD and AS in the LrGG-treated group, but did LrGG treatment affect the gut microbiome compared to placebo? The short answer is "yes" in early life, but with little rhyme or reason. Specifically, at three months of age, *Bifidobacterium longum* was underrepresented in the *Bifidobacterium* genus in participants who went on to develop ADHD or AS by age 13, and *Bifidobacterium* as a whole was deficient in that population at six months. At 18 months, *Bacteroides* and *Lactobacillus-Enterococcus* group bacteria were lower in those who ultimately developed neuropsychiatric disorders (recall that the supplemented bacterium was from the genus *Lactobacillus*). At 24 months of age, those who went on to develop ADHD or AS tended to have lower counts of *Clostridium histolyticum*. Finally, at age 13, there were no significant differences seen in the gut microbiome between children with ADHD and AS, compared to children without these disorders. There's a chance that these seemingly random differences in the gut microbiome through time may actually be random though, since the authors made many comparisons and didn't seem to correct for them.

While supplementation with LrGG early in life was linked to lower incidence of ADHD and AS by age 13, there was no consistent difference in the gut microbiome between those who developed the disorders and those who didn't.

Supplementation with *Lactobacillus rhamnosus* GG during the first six months of life was associated with a lower risk of developing ADHD or Asperger's by age 13. Although there was no difference in the gut microbiome between children who had the disorders and those who didn't at age 13, there were some minor, inconsistent differences early in life.

What does the study really tell us?

The authors describe this study as being a "preliminary and initial observation."

This should be emphasized, as there are quite a few limitations to this study.

One of the main limitations is "loss to follow-up." Recall that this study is actually a follow-up to a previous study. That initial study was not designed to see whether probiotics prevent ADHD and AS after 13 years, but instead to see whether six months of supplementation could prevent allergic hypersensitivity after two years. During the gap between the original study and this follow-up, around half of the subjects from the original study were lost. This phenomenon of "loss to follow-up" presents a large problem in interpreting the results, since we don't have any idea whether or not these patients developed ADHD or AS. And if some of them did, especially in the placebo group, this could dramatically affect the results, as shown in Figure 2 One rule of thumb suggests that having a loss to follow-up of more than 20% is a serious problem. In this study, the loss was about 50%.

There is also some evidence to suggest that the unobserved placebo group may have a higher risk of neuropsychiatric issues. Recall that the children who participated in the 13-year follow-up had significantly more months of exclusive breastfeeding than those who were lost to follow-up. Some observational evidence suggests that breastfeeding may have a protective effect against ADHD and childhood conduct disorders. So while there were few differences in the measured variables between those children which participated in this 13-year follow-up and those who didn't, the difference in the length of exclusive breastfeeding between those who were included in this study and those who were not may be enough to introduce serious confounding.

The difference in breastfeeding early in life between those who participated in the 13-year follow-up and those who didn't may have also confounded the findings concerning the gut microbiome. Breastfeeding is known to affect the gut microbiome, increasing the amount of bifidobacteria in the gut. Since breastfeeding affects the composition of the gut microbiome, and there was a difference between those included in the study and those who were lost to follow-up, the microbiome data presented in the study may be confounded as well.

It should also be noted that there was a statistically *non*-significant difference in the length of exclusively breastfed months in the children who were analyzed at age 13: those children who went on to develop ADHD

Figure2:- Why loss to follow-up is so important

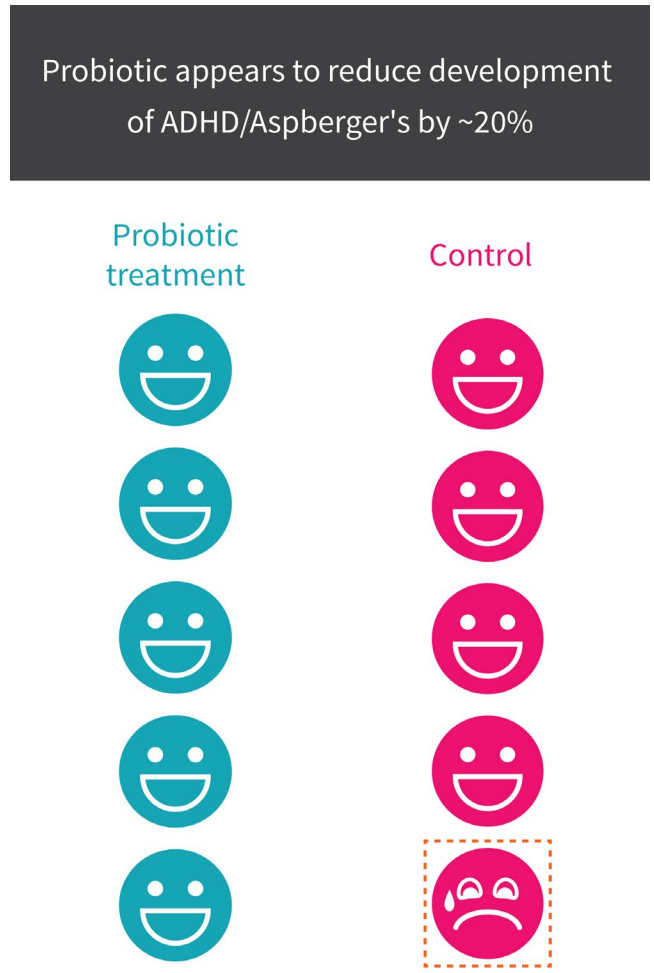
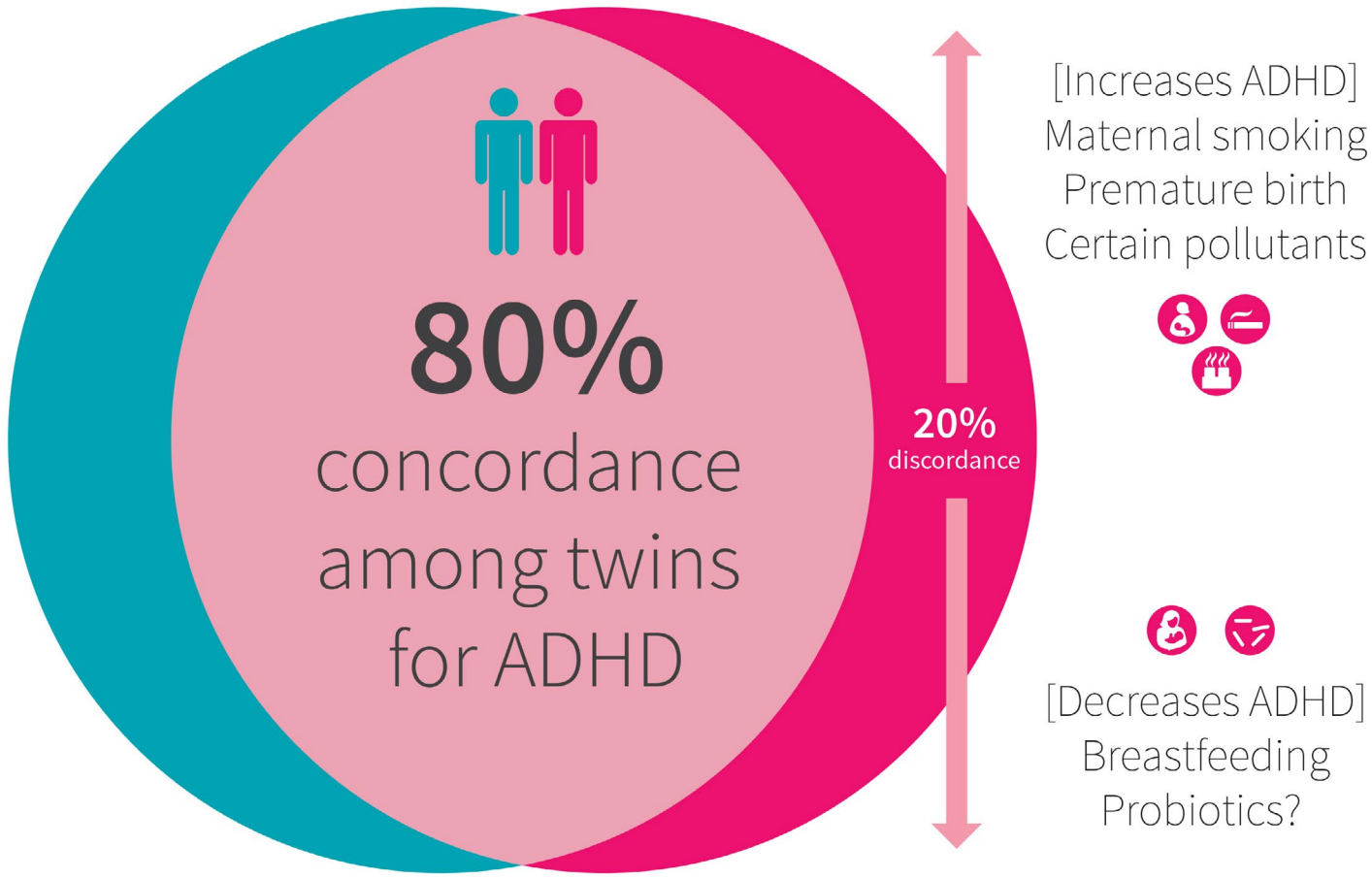


Figure 3: Environmental and epigenetic determinants of ADHD



or AS were breastfed exclusively for two months on average, compared to three months on average for the healthy children. Because of the smaller sample size in the follow-up population, this difference was not statistically significant ($p = 0.16$). However, given that breastfeeding may impact both the microbiome and the risk of behavioral disorders later in life, it would have been interesting to see whether LrGG supplementation would have still have a significant impact on ADHD/AS development if length of breastfeeding was taken into account. Unfortunately, the authors did not provide this analysis.

Finally, it's important to keep in mind that this study was actually a follow-up of previous study that was designed to look at probiotics' effects on allergic hypersensitivity. The original study was not designed to look at ADHD and AS. For this reason, the results of this study, while useful to guide further research, should be considered preliminary.

The results of this study should be taken with a grain of salt due to a large loss of participants over time and possible confounding issues.

The big picture

Both ADHD and the autism spectrum disorders such as AS have strong genetic components. However, environmental factors could influence the development of these disorders. The "nature versus nurture" aspect of ADHD specifically is depicted in Figure 3.

Environmental effects associated with an increased risk of ADHD include maternal smoking, premature birth, and exposure to environmental toxins such as lead and polychlorinated biphenyls (PCBs). Diet has also been examined in the context of mitigating existing symptoms, although with mixed results, in part due to low study quality. There is little evidence concerning the role of dietary supplementation in reducing the risk of developing ADHD or AS in the first place though, which is partly what makes this study novel and interesting.

The idea that omega-3 fatty acids may influence the development of both ADHD and the autism spectrum disorders has recently been gaining traction, though. One omega-3 fatty acid, docosahexaenoic acid (DHA), accumulates in the fetal brain throughout gestation and continues to build

during the first two years of life, suggesting that early supplementation may be useful. Furthermore, higher levels of serum DHA, which is present in breast milk, is correlated with higher childhood cognitive function and decreased hyperactivity and impulsivity. Preliminary evidence also suggests that DHA levels inversely correlate with the risk of autism. These facts may partially account for the increased risk of ADHD in premature birth mentioned above, as well as the possible protective role of breastfeeding as mentioned in the last section. Indeed, a recent case control study has found that omega-3 fatty acid deficiency may be a risk factor for both ADHD and autism spectrum disorders.

While the research is still young, studies like this one provide interesting hypotheses concerning the role of supplementation in the prevention or amelioration of developmental disorders, which can be tested through additional studies.

While genetics seems to play a significant role in the development ADHD and Asperger syndrome, recent preliminary evidence suggests that dietary supplementation could hypothetically impact the risk of developing these disorders. More rigorous studies are needed to confirm these ideas.

Frequently asked questions

Isn't it weird that the researchers supplemented breastfeeding mothers? How could that influence their child's gut microbiome?

It is a little weird. The rationale the authors gave in the original study for this protocol was that giving the probiotics to infants directly or to their nursing mothers "have resulted in similar amounts of Lactobacillus GG in infant faeces." However, the citation they provide to back up this statement doesn't clearly support this, as only ranges were given with no statistics like standard deviation or means, and no statistical tests which show that the difference between groups was not statistically significant. Furthermore, the lower end of the range of fecal counts in breastfed infants whose mothers received the supplement for a month was only 103 colony forming units of LrGG per gram, as opposed to 4.0 × 107 in those infants who directly took the supplement, which is a 40,000-fold difference. So, this doesn't shed a whole lot of light on the matter. Furthermore, no explanation of why the counts would be similar or the mechanism by which supplementing nursing mothers with LrGG could directly affect LrGG counts in their breastfed infants could be found.

> "Both ADHD and the autism spectrum disorders such as AS have strong genetic components. However, environmental factors could influence the development of these disorders."

However, a recent clinical study has shown that supplementing breastfeeding mothers both before and three months after birth with a probiotic mixture did increase the

levels of LrGG found in the infants early in life compared to mothers who were administered a placebo. Whatever the mechanism, it seems like probiotic supplementation of breastfeeding mothers may influence some aspects of their child's microbiome.

All of the children who developed AS and ADHD in this study were male. Are males more at risk for these disorders?
Yes. Both disorders are more prevalent in males. Males are roughly four times more likely to develop autism spectrum disorders. In fact, all four initial cases described by Hans Asperger were males. Roughly three times as many males get ADHD than females.

The original study from these authors was about the effects of probiotic supplementation on allergic hypersensitivity. Were the results of that original study promising?
Yes. The risk of atopic eczema, the main allergic outcome, was halved with probiotic supplementation. Furthermore, the risk was mitigated in both infants whose mothers took LrGG while breastfeeding, as well as for those infants who were directly administered the probiotic.

All of the children included in this study were at risk for allergies. Could this have affected the risk of these children developing ADHD or AS at all?
Maybe. Bronchial asthma and allergic rhinitis may be associated with an increased risk of ADHD, leading some to hypothesize that ADHD could be in part due to hypersensitivity to environmental triggers. In addition, food allergies may be more prevalent in children with autism spectrum disorders. So it's possible that this population of children may have had an increased risk of developing ADHD or AS, although more research needs to be done on this topic.

What should I know?

This randomized, placebo-controlled study found that supplementation with *Lactobacillus rhamnosus* GG in the first six months of life attenuated the development of ADHD or Asperger syndrome by age 13. However, these results should be considered preliminary due to large loss to follow-up and the presence of confounding. Further trials, which lack these limitations, are needed to confirm the results. ◆

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> "Males are roughly four times more likely to develop autism spectrum disorders. In fact, all four initial cases described by Hans Asperger were males. Roughly three times as many males get ADHD than females."

The gut microbiome's role in type I diabetes

The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes

Introduction

There is a lot of evidence to suggest the microscopic bacteria in our gut, known as the microbiome, plays a large role in health. The ERD has discussed some of these roles in past issues (see: "Of mice and guts (and exercise performance)" and "Gut bugs and fiber" in ERD #2, and "A mouse's microbiome may cause its brain to leak" in ERD #3). Some of the diseases in which the microbiome may be involved include irritable bowel disease, rheumatoid arthritis, and even obesity. Recent evidence suggests that type I diabetes may be added to the list.

The types of diabetes

The term "diabetes" comes from an ancient Greek work meaning "to go through" or "to siphon." The disorder was named for one of its cardinal symptoms: frequent urination. When diabetics drink liquids, they tend to "go right through them."

There are actually several types of diabetes. Diabetes insipidus is a disorder characterised by frequent urination that can be traced back to how the body responds to a hormone known as antidiuretic hormone. However, this disorder has little in common with what most people refer to when they say "diabetes," which is formally known as diabetes mellitus.

"Mellitus" comes from the Latin word for "honey," which refers to the fact that people with diabetes mellitus have urine with sugar in it. There are two types, both of which have to do with insulin (which helps the body deal with sugar, hence the "sweet" urine). Type I diabetes mellitus, previously known as insulin-dependent diabetes or juvenile diabetes since patients often develop it early in life and require insulin injections, is an autoimmune disorder characterized by the body's inability to produce insulin. Type II diabetes mellitus, previously known as adult-onset or insulin-independent diabetes since patients often develop it in adulthood and do not usually need insulin injections to live, is primarily characterized by insulin resistance, where the body can usually produce insulin but has trouble responding to it.

Type I diabetes is an autoimmune disorder, meaning that a person's immune system attacks their own body. In the case of type I diabetes, the immune system specifically attacks the insulin-producing beta cells of the pancreas, which reduces or eliminates the person's ability to regulate their own blood sugar. The typical symptoms of type I diabetes include excessive thirst, frequent urination, and excessive hunger. The first and second occur because of high blood sugar, which pulls water from out of the body and into urine as it's filtered by the kidneys. The third symptom is due to varying factors, such as body tissue's lack of ability to use sugar in the blood as fuel (since insulin is no longer being produced in sufficient quantities), in addition to shrinking fat stores caused by the condition leading to declining leptin, which provokes hunger. This condition can lead to death if not managed through insulin, and its long-term complications include kidney, heart, eye, and circulation problems.

Most people (70%) who develop type I diabetes tend of have certain kinds of human leukocyte antigen (HLA) genes, which are major proteins that help regulate immune function. At first glance, type I diabetes looks largely like a genetic disorder. The weird thing, though, is that only about 5% of children who have these HLA genotypes which put them at risk actually develop type I diabetes. This strongly suggests that more than one gene impacts this condition, and/or something beyond genetics influences the development and progression of the disease. But what could that something be?

Animal studies point to the gut microbiome as a possible culprit. Studies conducted in diabetes-susceptible mice show that completely knocking out both copies of a gene important for immune system microbe recognition results in the prevention of diabetes development. Furthermore, mice with only one of the two copies of the gene knocked out, which usually go on to develop diabetes, are protected from developing the disease if their guts are colonized by healthy microbes. This indicates that an immune response to certain species of gut microbiota may play a role in the development of type I diabetes, at least in mice.

But what about humans? There is evidence to suggest that the composition of the gut microbiome differs between children who have anti-pancreatic antibodies and those without. This doesn't say much about whether the microbiome changed in response to the diabetes, or whether it developed before the diabetes, though. To answer that question, a prospective study of how the gut microbiome changes through time was conducted.

Type I diabetes is a partially genetic disease, but has a strong non-genetic component. This study was designed to determine whether the gut microbiome may play a role in the disease's development.

Who and what was studied?

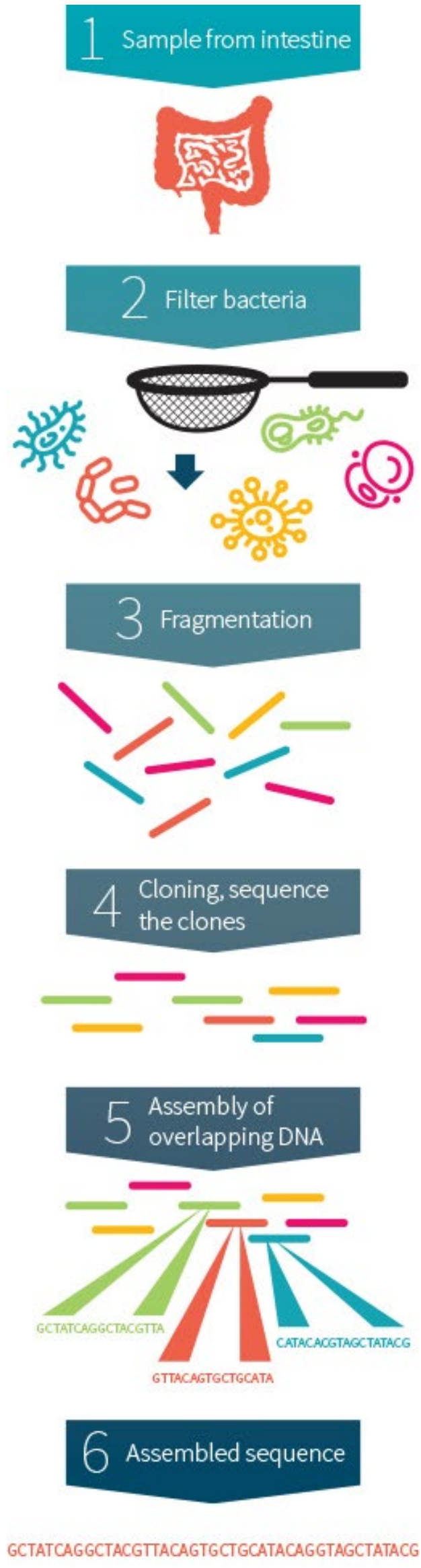
This study involved a group of infants from Estonia and Finland, recruited at birth. All of them were very likely to develop type I diabetes early in life based on their HLA genotype. Stool samples from the infants were collected monthly by the parents and sent to the researchers for approximately three years. That's a lot of poop, but out of volumes of poop came volumes of data about what was going on inside the guts of the infants as some of them went on to develop type 1 diabetes.

There are many different species of bacteria in stool, all doing different things biochemically, and producing different metabolites. The researchers were able to examine this big and complicated picture using metabolomics and shotgun metagenomics. The various "-omics" are the study of large and complex samples of biological molecules. Metabolomics was described in the ERD #5 article "Can you go too nutty over pistachios?" but basically involves identifying specific biochemicals in a sample. Shotgun metagenomics (shown in Figure 1) allows for identification of unique segments of the DNA from a population of different organisms, which allows for identification of both the organism and their unique biochemical profiles. Thus, shotgun metagenomics can tell us both about what organisms are in the sample as well as what they can do biochemically.

By applying these techniques to stool samples throughout the three year study period, a very detailed picture of how the infants' gut microbiome changed through time emerged. In addition, serum samples were taken and metabolomics were performed on these as well. Dietary and environmental variables were also measured to see what other factors may have contributed to changes in the infants' microbiome.

During the course of the study, some of the infants developed antibodies against their own pancreas, which was expected since all the infants were at risk for type I diabetes. Stool and blood samples from infants who developed antibodies against their own pancreas during the course of the study (called "seroconverters," since their serum converted to car-

Figure 1: Shotgun metagenomics



rying these antibodies) were matched with the samples of non-seroconverting infants. This allowed the researchers to compare the microbiomes of seroconverted infants with those who did not seroconvert to see what differences, if any, there were between the two groups.

Infants at risk for type I diabetes were followed from birth for three years to see how their gut microbiomes changed as autoimmune antibodies and type I diabetes developed.

What were the findings?

The authors were able to get quite a high-resolution view of how the microbiome of these infants developed over time, and found two general trends concerning how the microbiome developed over time in all the infants. First, the diversity of the microbiome increased exponentially over time, peaking at the end of the study. However, those

> “ […] although the bacteria became more diverse with time, they were doing similar things biochemically. ”

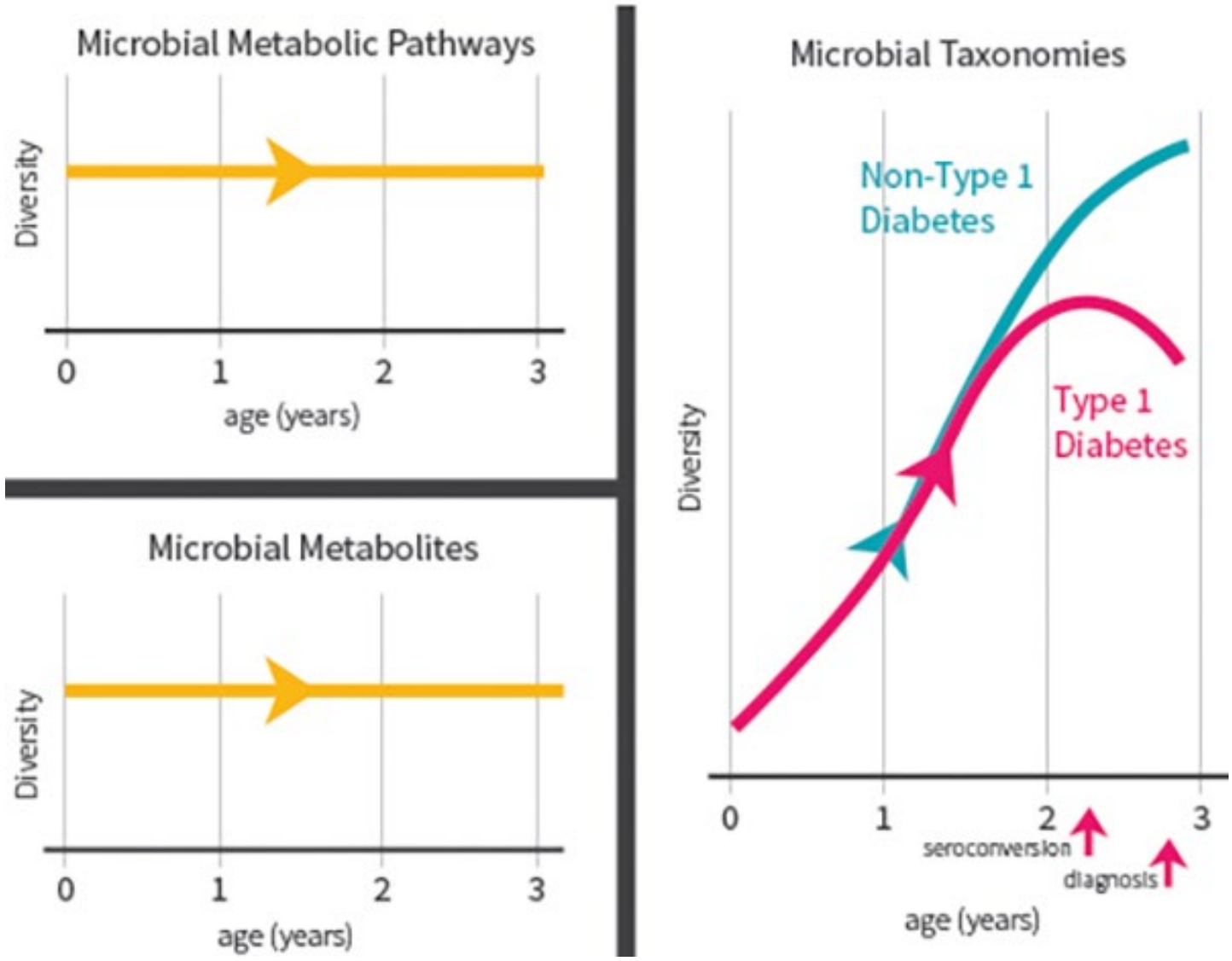
species which were abundant at birth tended to remain present and constant throughout each infant's development. Second, even though the bacteria themselves grew in diversity over time, the metabolites that the bacteria produced in the gut and the biochemistry those bacteria were capable of remained remarkably stable throughout the course of the study. Thus, although the bacteria became more diverse with time, they were doing similar things biochemically.

When subgroups of the infants were examined, some interesting patterns also emerged. Breastfeeding infants led to increased levels of *Bifidobacterium* and *Lactobacillus* species, some of which are being investigated for their probiotic properties. Also, the Estonian infants had higher levels of Bacteroides and Streptococcus species, some of which may be pathogenic. This suggests that nationality is correlated with the microbiome. Ethnicity, specific geographic location, and related factors (such as differing diet by locale) may also be correlated with the microbiome, and should be taken into consideration when interpreting observational genetics-based studies such as this.

Diet also seemed to affect the microbiome, but mildy. Finally, there was no difference seen in the microbiome of infants who were delivered vaginally versus cesarean section, although the sample of infants who were delivered via C-section was small, so perhaps larger samples would reveal a difference.

But what about the microbiome's relation to type I diabetes? Out of the 33 infants, 11 seroconverted. Out of the seroconverted infants, four developed type I diabetes during the course of the study. As seen in Figure 2, the researchers found that there was a drop in diversity of the microbiome in those infants who went on to develop diabetes during the study period. This drop preceded the diagnosis itself, but occurred after these infants seroconverted. Curiously, both seroconverters who didn't develop diabetes during the study period and nonconverters experienced the exponential increase in microbial diversity described earlier. This drop in diversity was accounted for by an increase in species from Ruminococcus and Streptococcus, both of which are possibly pathogenic, along with a decrease in certain species

Figure 2: Microbial diversity in guts of study infants



Adapated from Kostic et al., Cell Host & Microbe, 2015

which tend to be depleted in inflammatory states.

The metabolic pathways used by the bacteria were also correlated with type I diabetes. Bacteria in the infants who developed the disease tended to make less nutrients for themselves and increased their passive absorption of nutrients. This suggests an inflammatory state in the gut, where the bacteria were able to feed off of the nutrients from inflamed, dying tissue. Increased human beta-defensin 2, a marker of gut inflammation, was also seen in those infants who went on to develop diabetes, which also suggests that inflammation is playing a role here. An increased amount of triglyceride and branched-chain amino acid production was also observed in diabetic infants, which were correlated with increased amounts of the microbes *Blautia* and *Ruminococcus*. This is bad news, since high triglycerides are associated with kidney problems and poor glycemic control in type I diabetes patients, and branched-chain amino acids are associated with worse diabetic control. Thus, it's possible that changes in the microbiome may hasten the development of type I diabetes in these infants.

The microbiome generally tends to increase in diversity with age, but becomes less diverse in those infants who develop type I diabetes. This change precedes diagnosis of the disease, but occurs after seroconversion.

What does the study really tell us?

This study gives an extremely detailed, unprecedented look at changes to the microbiome of infants predisposed to type I diabetes. It found that microbiome diversity decreased prior to type I diabetes, while inflammation and possibly pathogenic species increased, and that these changes only occurred only in infants who went on to develop the disease

during the course of the study. In both infants who developed autoimmune antibodies against the pancreas but didn't develop diabetes and infants who didn't make any autoimmune antibodies, no such decrease in diversity occurred; instead, their microbiomes kept on increasing in diversity.

But behind all of the "-omics" and impressive techniques used, this is still an observational study. That means it's hard to determine causality. Did the increase in pathogenic species in the microbiome cause diabetes in the infants, or did the infants' inflammation during progression to diabetes simply create an opportunity for these pathogenic species to thrive? We can't tell from this study alone. The fact that the decrease in microbiome diversity and increase in potentially pathogenic species occurred before diabetes developed, and not in those who didn't, is at least consistent with causality, but there's no sure-fire way to tell. The applicability of this study to infants in general may be limited, as the sample consisted of infants in Finland and Estonia, and as such isn't likely to be very diverse genetically.

However, this study definitely opens the door to test some hypotheses further down the road. Probiotics are being investigated in newborns and children, and perhaps interventional studies in infants predisposed to type I diabetes may yield promising results. Also, antibiotics have been shown to partially prevent type I diabetes in mice. Since this study helps identify some of the specific species associated with the development of diabetes, it opens the door for testing targeted antibiotic therapy to prevent or delay diabetes onset in susceptible infants.

The microbiome changes in the gut preceding type I diabetes in susceptible infants are consistent with causality, but causality cannot be proven here since this is an observational study.

The big picture

Colonization of the gut by the microbiome actually begins while the fetus is still in the womb, and is strongly impacted by microbial exposure during birth, as seen in Figure 3. This study showed that the normal microbiome generally increases in diversity as an infant ages, and that a decrease in diversity precedes the development of type I diabetes.

"The applicability of this study to infants in general may be limited, as the sample consisted of infants in Finland and Estonia, and as such isn't likely to be very diverse genetically. However, this study definitely opens the door to test some hypotheses further down the road."

This pattern of decreasing microbial diversity being associated with disease has also been noted in other diseases, including Clostridium difficile-associated diarrhea, Crohn's disease and ulcerative colitis, and obesity. It is looking more and more likely that diversity of the microbiome is important for, or at least associated with, health.

In the case of type I diabetes, there is a large variability in onset of diabetes after seroconversion, ranging from weeks to decades. It is unclear why this is the case. This study hints that the gut microbiome could play a role in onset, although it can't be said for certain given the study's observational nature. And it may not be the only factor. One model for the development of type I diabetes suggests that a "perfect storm" of three factors need to coincide for diabetes to develop in genetically susceptible individuals: an altered microbiome, a leaky and permeable gut, and an aberrant immune response. So, while this study provided some very detailed insights into one of these factors, there's still a lot left to be learned.

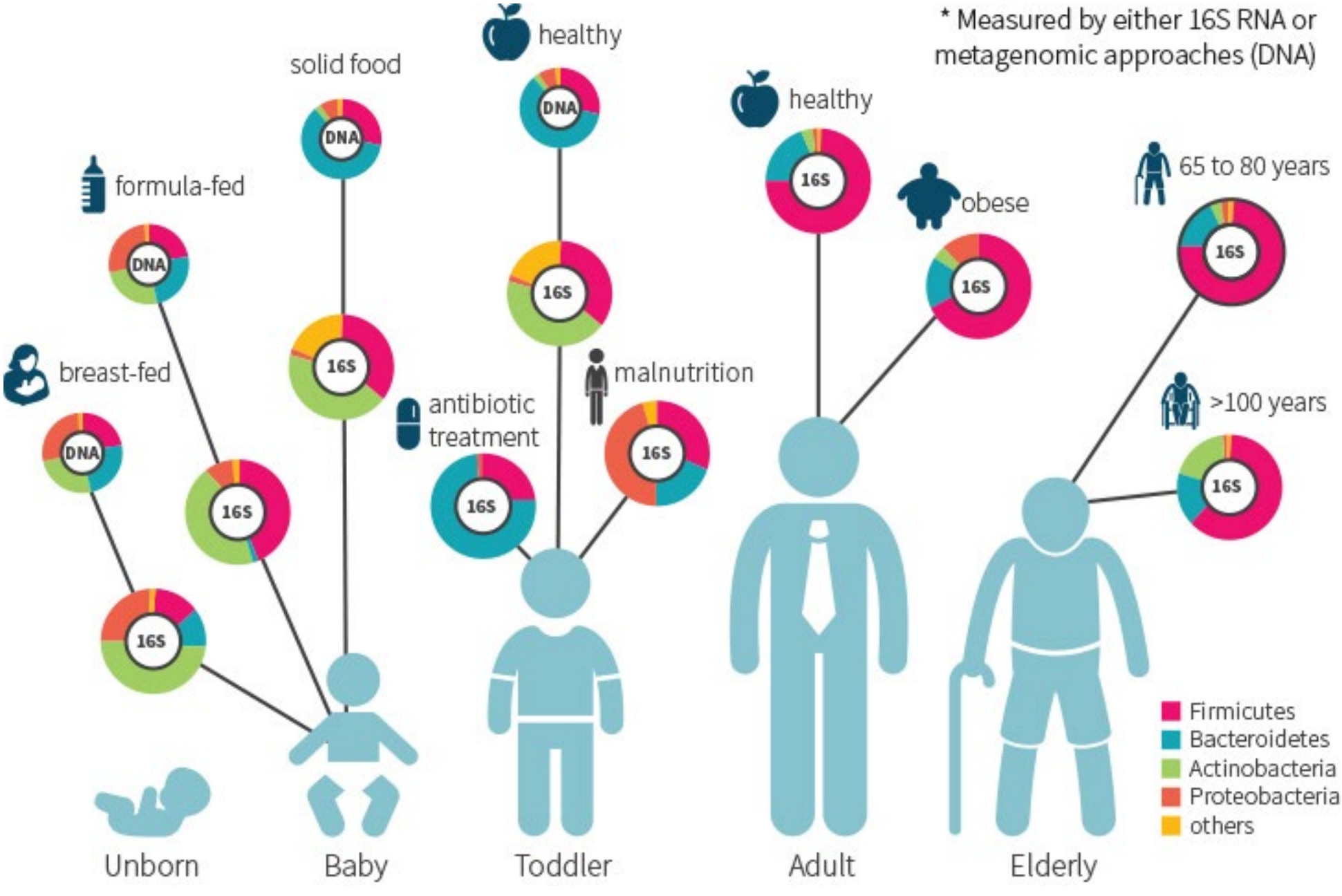
Decreased diversity of the microbiome has been observed in other diseases in addition to the onset of type I diabetes. And while the microbiome may be important in the development of diabetes, there are other pieces of the puzzle still left to explore.

Frequently asked questions

Is breastfeeding good for microbial diversity?

The research is still young on this, but one study found

Figure 3: Human gut microbiome changes, from birth to old age



Source: Ottman et al., Front Cell Infect Microbiol, 2012

that exclusively breastfed infants actually exhibited a lower microbiome diversity than infants who were not exclusively breastfed. However, exclusively breastfed infants did have lower levels of possibly pathogenic bacteria, and may have been able to transition to solid foods more easily.

What about antibiotics? How do they affect infant microbial diversity?

Antibiotic therapy reduces microbial diversity, with more intensive therapy having a larger impact. The longer-term consequences of this impact are not completely understood, however. Antibiotic use in the first year of life has been linked to inflammatory gastrointestinal conditions in later childhood, although the evidence here is also observational and can't prove causation.

Is there any clinical evidence that probiotic supplementation could help type I diabetes?

There are case reports suggesting efficacy, but these are not strong evidence since confounding factors are present and there was a lack of controls. A clinical trial is in the works, but the results have not yet been released.

What about type II diabetes? Any clinical evidence there?

There's not much evidence, but it is a little stronger. One double-blind, placebo-controlled trial found that probiotic yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium lactis* over a six week period reduced fasting plasma glucose and hemoglobin A1C in people with type II diabetes. Another study found that insulin sensitivity was preserved in men with type II diabetes supplementing *Lactobacillus acidophilus*, compared to a control group over four weeks.

What should I know?

Gut microbiome diversity steadily increases from birth onward, although major species in the microbiome tend to establish themselves early and stick around as the infant ages. However, diversity sharply decreases, and potentially pathogenic organisms increase, prior to the development of type I diabetes in infants genetically predisposed to the disease. This is consistent with the hypothesis that the gut microbiome may partially cause type I diabetes in susceptible infants. However, causality cannot be strictly demonstrated here, since this was an observational study. ◆

Contribute to the increasing diversity of opinions about this article over at the ERD private Facebook forum!

> "Antibiotic use in the first year of life has been linked to inflammatory gastrointestinal conditions in later childhood, although the evidence here is also observational and can't prove causation."

I get by with a little help from my friends: probiotics and depression

A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood

Introduction

For thousands of years, clinicians have observed a connection between the gut, brain, and overall health. Hippocrates is famously quoted as saying "all disease begins in the gut." This should not come as a surprise to anyone who has ever experienced gastrointestinal (GI) symptoms like diarrhea, indigestion, or abdominal discomfort in response to changes in their emotional state. Studies in both healthy patients and in people with functional bowel disorders have confirmed connections between emotional state and GI function.

The gut and brain communicate through neural, endocrine and immune pathways. It has become increasingly clear that interactions with intestinal microbiota are also an important part of this communication. A number of animal and human studies have examined the relationship between gut bacteria and mood symptoms such as anxiety and depression, leading to the idea that probiotic supplementation may be a potential strategy for reducing or preventing depression.

According to the cognitive theory of depression, an individual's negative and distorted thinking is the basic psychological problem at the root of depressive syndrome. Cognitive reactivity refers to the activation of dysfunctional patterns of thinking triggered by subtle mood changes. This is a key feature in the development and occurrence of depression, and as such would be a relevant target for interventions. Dysfunctional patterns of thinking can include thoughts of hopelessness, thoughts of hurting oneself or others, ruminating on the causes and consequences of anguish, and a general loss of motivation for life. These responses are thought to come from underlying negative thought patterns that get brought to the surface during times of low mood. Cognitive reactivity appears to be a cause of, rather than simply an association with, depression, since higher cognitive reactivity scores precede and predict the onset of depression, even in people with no prior incidence of depression.

Considering the vast potential to treat and prevent mood disorders by improving gut health, the objective of this Dutch study was to determine the effects of a probiotic supplement on cognitive reactivity to sad mood, as well as symptoms of depression and anxiety in non-depressed, healthy adults.

"Cognitive reactivity appears to be a cause of, rather than simply an association with, depression, since higher cognitive reactivity scores precede and predict the onset of depression, even in people with no prior incidence of depression."

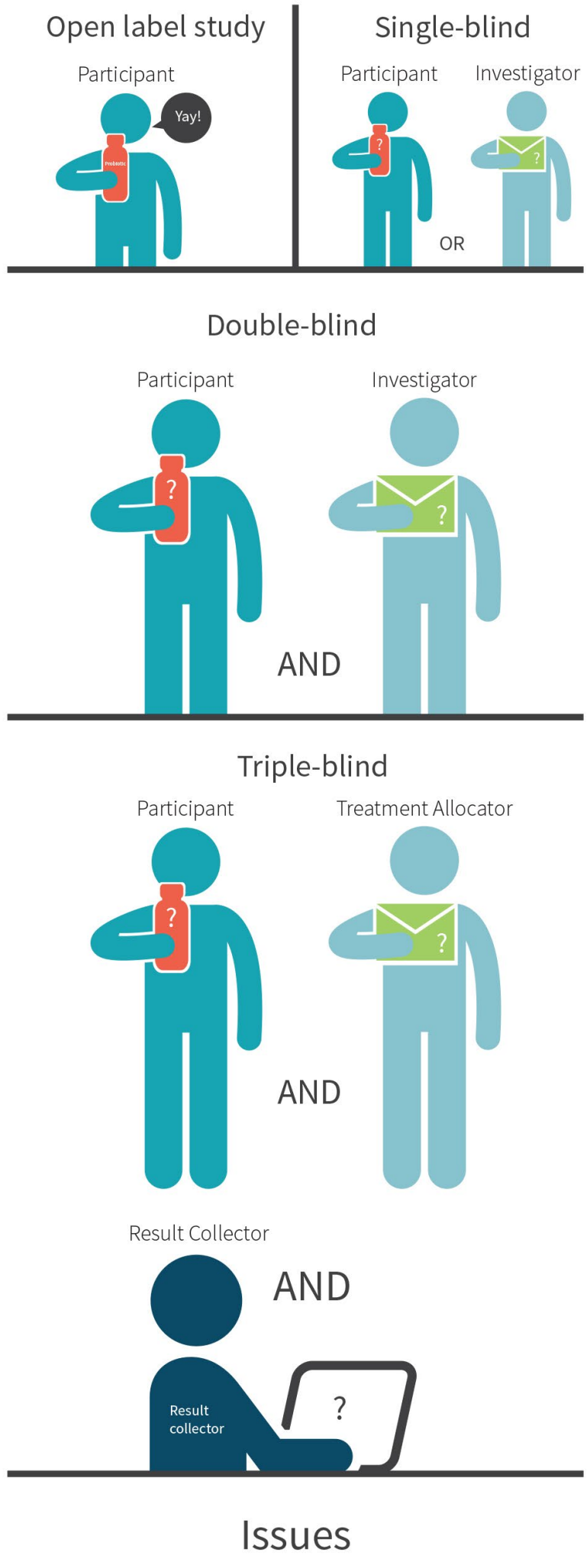
Cognitive reactivity, which involves negative thought patterns triggered by mood change, has been shown to predict the onset of depression. Since the brain and gut communicate through several pathways and the gut microbiome has recently been found to influence this communication, it is possible that probiotic supplementation could affect cognitive reactivity. This is what this study set out to test.

Who and what was studied?

Forty healthy, normal weight college-age adults participated in this triple blind study. A triple blind study means neither the participants, researchers, nor the people who organize and analyzed the data knew which group a participant was in. Different types of study blinding are depicted in Figure 1. The participants did not smoke, had no reported medical conditions, food allergies, medications, or drug use, and consumed no more than three to five drinks per week. Participants also did not have any psychiatric or neurological disorders, or any personal or family history of depression or migraines. They were randomly assigned to receive either a probiotic supplement (n=20, five males) or placebo (n=20, three males) for four weeks. The menstrual cycle was not controlled for in the female participants.

Interestingly, all participants were told they were receiving the probiotic supplement. This is different from many other trials, as participants would normally be told they have an equal chance of receiving the supplement being studied or placebo. The probiotic used was a mixture that is commercially available in the Netherlands, containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *L. casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* (W19 and W58). The participants consumed either supplement or placebo for four weeks.

Figure 1: Triple blinding vs other blinding



*Definitions are not standard. Sometimes the other blind is an investigator rather than treatment allocator, for example.

**The term "Masking" has been proposed, given the number of people with serious visual impairments (e.g. blindness)

Three different questionnaires were used before and after the intervention period to quantify the outcomes. The revised Leiden Index of Depression Sensitivity (LEIDS-r) measured the perceived cognitive reactivity to transient changes in sad mood, which indicates vulnerability to depression. The LEIDS-r is made up of 34 questions that assess the extent to which dysfunctional thoughts are activated when someone is experiencing a mild state of dissatisfaction with their life. Example questions include "*when in a low mood, I take fewer risks*," or "*when in a sad mood, I more often think about how my life could have been different*." Responses are given on a 5-point scale, with 0 being "does not apply to me" and a score of 5 meaning "very strongly applied to me." The scale measures vulnerability to depression overall, and consists of six different subscales: regard to aggression, hopelessness/suicidality, acceptance/coping, control/perfectionism, risk aversion, and rumination.

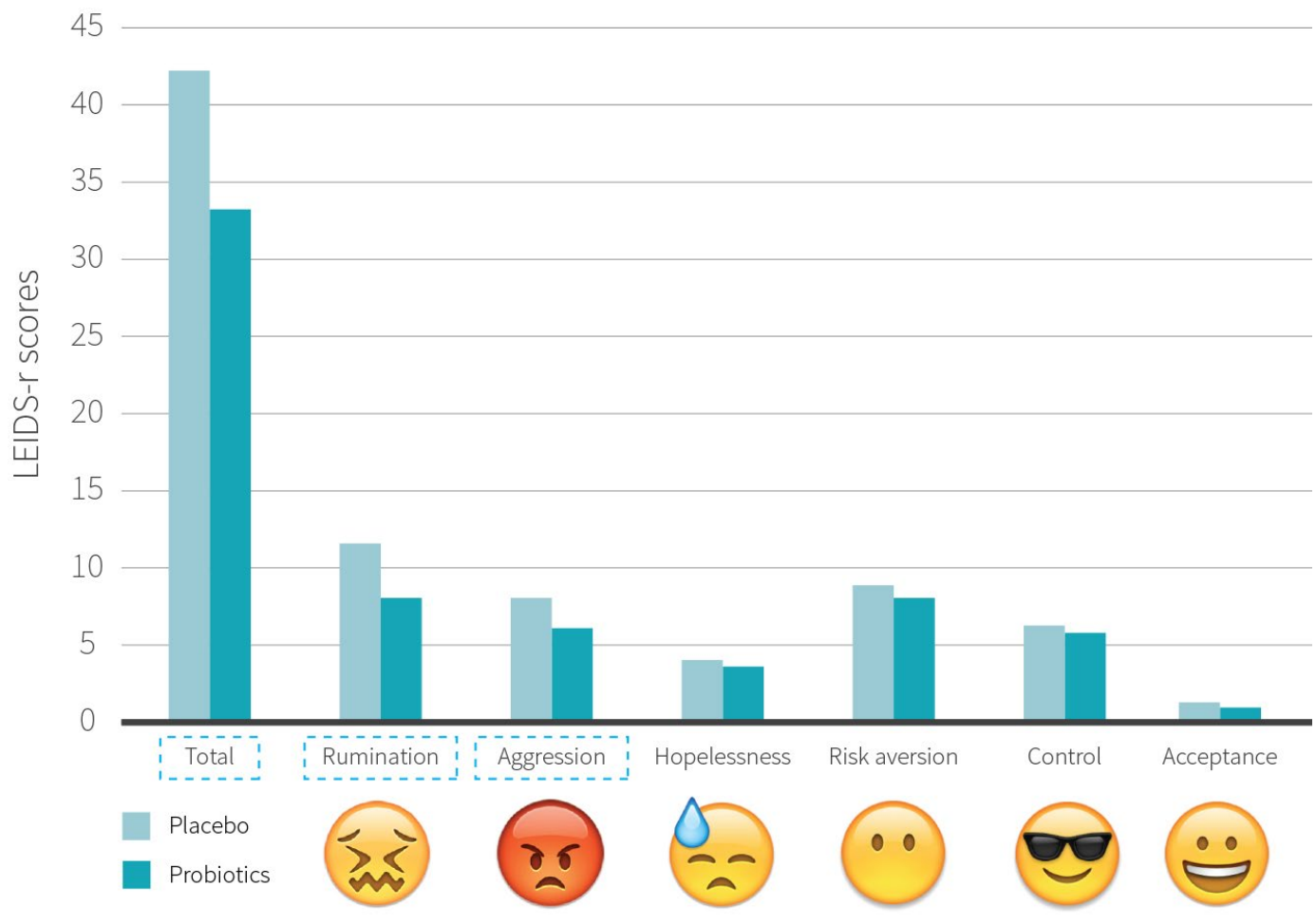
The Beck Depression Inventory II (BDI-II) is a 21-item questionnaire that assesses the existence and severity of depressive symptoms occurring during the previous two weeks. Similar to the LEIDS-r, questions are rated on a 4-point scale ranging from 0 to 3 in terms of severity, and the total score is added up to classify the level of depression (minimal, mild, moderate, or severe). The Beck Anxiety Inventory (BAI) is also a 21-item questionnaire, used to assess the existence and severity of anxiety symptoms occurring during the previous week. Questions are also answered on a 4-point scale ranging from 0 to 3 in terms of severity.

Forty healthy college-age adults with no personal or family history of depression took either placebo or a probiotic mixture. Cognitive reactivity was measured with the LEIDS-r questionnaire before supplementation began and after four weeks of supplementation. Anxiety and depression were also measured using questionnaires.

What were the findings?

The study findings are summarized in Figure 2. None of the participants (in either group) showed any signs

Figure 2: Study results



of depression (using the BDI-II) or anxiety (using BAI) at baseline or follow-up. Differences were seen on the LEIDS-r, however, which measures vulnerability to future depression. Participants who received the four-week probiotic supplement showed a significantly lower score for overall cognitive reactivity to sad mood, mainly accounted for by reduced rumination and aggressive thoughts. No differences were found between groups for hopelessness, control, risk aversion, or acceptance.

What does the study really tell us?

"*The present results indicate, for the first time, that probiotics intervention can influence cognitive mechanisms that are known to determine vulnerability to mood disorders.*"

This study set out to determine the effects of a multi-species probiotic supplement on cognitive reactivity, an important marker in predicting future depression, in healthy young men and women without any personal or family history of mood disorders. Though none of the participants showed any signs of current anxiety or depression, a four-week probiotic intervention showed significantly reduced cognitive reactivity scores, suggesting a reduced vulnerability to future depression. Reductions in total cognitive reactivity score were largely due to decreases in the aggression and rumination subcategories. This is relevant because the people who ruminate about the causes of being in a sad mood may have a harder time recovering from depression.

These participants were healthy and had no diagnosable anxiety or depression at baseline, so improvements in these scores would not necessarily be expected. The lack of any existing mood disorders in the participants is important because it allows the researchers to test for any influence on *future* depression, which the LEIDS-r questionnaire has been shown to do. Of course, further long-term studies using the probiotic interven-

> "Participants who received the four-week probiotic supplement showed a significantly lower score for overall cognitive reactivity to sad mood, mainly accounted for by reduced rumination and aggressive thoughts."

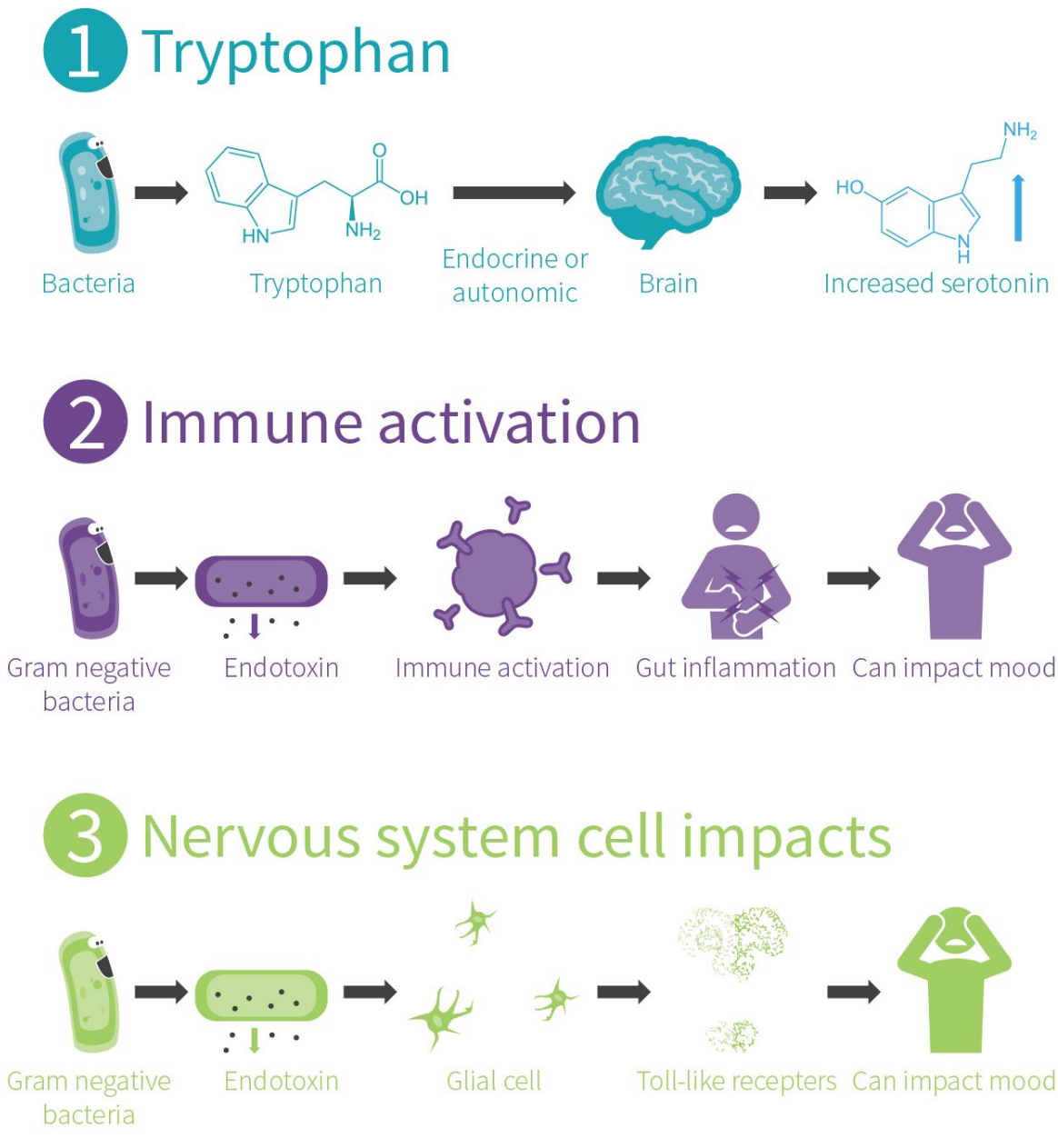
tion would be needed to confirm if these predictions become clinically relevant.

However, we can calculate a guesstimate of the odds reduction of developing depression based on the LEIDS-r score. The roughly nine-point reduction in the LEIDS-r score seen in this study due to probiotic supplementation translates to an odds ratio of 0.76, in terms of developing depression over a two-year period, based on previous research.

While no mechanisms of action were studied, a number of hypotheses can be considered, which are shown in Figure 3. Cognitive reactivity scores can predict the depressive response to serotonin depletion, and gut bacteria may increase serotonin in the brain by increasing plasma tryptophan levels. Decreased intestinal permeability from the probiotic supplementation could also play a role, as increased gut permeability can lead to symptoms of depression. A review of the effects of probiotic supplements on intestinal permeability found a positive effect in 48% of the controlled studies.

While compliance was not confirmed by stool analysis and dietary control did not include consideration for other probiotic-rich foods (i.e. yogurt), the biggest limitation in being able to draw wider conclusions from this study is the disproportionate female to male balance. Participants in this study were 80% female, which is opposite to the male gender bias often found in the scientific literature. This is relevant because men and women have different gut microbiomes due to differences in sex hormones, and we are also somewhat different both emotionally and cognitively. This is also a fairly small study in a young population, which makes generalization difficult. Although ethnicity wasn't

Figure 3: Three of the ways that microbiota can impact the brain



explicitly mentioned in this study, this study was conducted in the Netherlands, which may warrant caution when generalizing to broader populations As we've seen in ERD #6 in "The gut microbiome's role in type I diabetes," nationality and ethnicity can correlate with microbiome differences.

This study suggests that a multispecies probiotic supplement reduces cognitive reactivity, which is associated with a lower risk of future depression. This is plausible, since there are several mechanisms by which the gut microbiome may affect vulnerability to depression, although these mechanisms were not examined in the study under review. The small sample size and limited diversity in age, gender, and ethnicity makes the results difficult to generalize.

The big picture

A number of human and animal studies show reduced signs of depression and anxiety with probiotic supplementation, though improvements are often seen only with pre-existing anxiety or depression.

Taking a probiotic supplement made up of multiple strains of bacteria can have increased effectiveness through an additive or synergistic effect of the individual strains, compared with mono-species supplements. However, some probiotics may work in an antagonistic manner, so combinations of strains should be studied both individually and in combination before creating a multispecies product. A previous study by one of the co-authors of this study reported improvements in intestinal barrier function by each probiotic strain used in this study separately, as well as in the combined product. Another study that included that same co-author found a decrease in migraines during the second and third month of taking the same supplement. However, no control group was used and no placebo was given.

As mentioned in last month's ERD article on HMB supplementation, the fact that a company sponsors the research does not automatically taint the results. However, it is worth noting that the aforementioned study about intestinal barrier function was done by the company's own internal research and development scientists. Both the study about migraines, as well as the study under review, featured the same employee from Winclove Probiotics as a co-author. Despite this, the authors of this paper state that "no competing interests exist."

Conflicts of interest

Conflicts of interest occur when the people who design, conduct, or analyze research have a motive to find results that suit their needs. The most obvious source of a conflict of interest is monetary. Sometimes, such as in this paper and the vitamin K2 study reviewed in this issue of the ERD, possible conflicts may exist even though the authors claim there are none. But these authors aren't alone. One study suggests that nondisclosure of possible conflicts of interest is somewhat common.

However, having competing interests doesn't automatically negate the results of studies. For instance, one review of major cardiovascular trials found that conflicts of interest had no impact on the results.

Conflicts of interest must be evaluated carefully. Don't automatically assume that they don't exist just because they're not disclosed, but also don't assume that they necessarily influence the results if they do exist.

Many studies to date have suggested that probiotics may have an effect on anxiety and depression, especially in animals, although human trials were mostly conducted on populations with pre-existing anxiety or depression. Multistrain probiotics could be more beneficial than individual strains in some, but not all, cases. Ideally, research should compare single versus multi-strain supplementation.

Frequently asked questions

Would probiotic supplementation have the same effect in men and women?

This study recruited both men and women, though it was predominantly (80%) made up of women and results were not listed separately by gender. Other trials using probiotic supplements have found both similar and differing effects among men and women. It is difficult to say how this product would compare between genders.

Could the benefits of this supplement extend beyond depression and anxiety?

Previous research using the same supplement has shown improvements in gut barrier function and a reduction in migraines. Additionally, a number of the species used in this product (but different strains) have shown cholesterol lowering effects, as well as contributing to improved immune function.

What I should know?

This study showed that healthy individuals consuming a multispecies probiotic for four weeks experienced a reduction in cognitive reactivity scores, which are a marker of vulnerability to future depression. In particular, these reductions were characterized by reduced aggressive and ruminative thoughts in response to sad mood.

This trial could provide a basis for larger trials in more diverse populations, possibly also testing the efficacy of multi-strain versus single species probiotics. ◆

We'll certainly be covering more trials on the gut-brain axis in future issues of ERD. In the meantime, discuss the ones we've already reviewed over at the ERD Facebook forum.

> "Previous research using the same supplement has shown improvements in gut barrier function and a reduction in migraines."

One pro of probiotic drinks: mitigating harm from overeating

Probiotic supplementation prevents high-fat, overfeeding-induced insulin resistance in human subjects

Introduction

Probiotics are microorganisms that can integrate into the microbiota of our gut in a beneficial way. They are living organisms that can colonize the gastrointestinal (GI) tract, and potentially crowd out some harmful microbiotic species.

Gut microbiota interact with their host in a number of ways. One is by contributing to intestinal metabolism through excretion of their own enzymes into the GI lumen, or through their own metabolism, particularly the fermentation of some types of fiber into short-chain fatty acids (SCFAs). These SCFAs support uptake of positively charged ions, also known as cations (e.g., Ca, Mg, K, Na), and can be taken up and metabolized further in the body. Microbiota are also involved in folate and biotin biosynthesis.

Since our food contains hundreds of thousands, maybe millions, of biochemical compounds, and since microbiota changes metabolism and uptake of nutrients, different microbiota can induce different changes in the host body. The purported effects range from changes in feeding efficiency (i.e., how much food is taken up) in animals, levels of gastric upset, and immune function under stress to reducing antibiotic-induced diarrhea and improving resistance to opportunistic infections. Figure 1 shows some of the ways

that an overly-permeable gut lining, influenced by microbiota balance, can impact conditions such as type II diabetes.

Previous studies have shown that distinctive microbiological profiles are associated with poor metabolic function in people and animals with chronic conditions. A couple examples are shown in Figure 2. Furthermore, probiotics may help ameliorate metabolic dysfunction in the host. However, whether probiotic administration can prevent the impairment of glucose homeostasis induced by overfeeding in humans has not been studied. The purpose of this study was to answer that question.

Gut microbiota play a large role in health, including potentially in metabolic disorders associated with diabetes and obesity. The goal of this study was to determine whether probiotics that influence the microbiota could help prevent glucose impairment induced by overfeeding in humans.

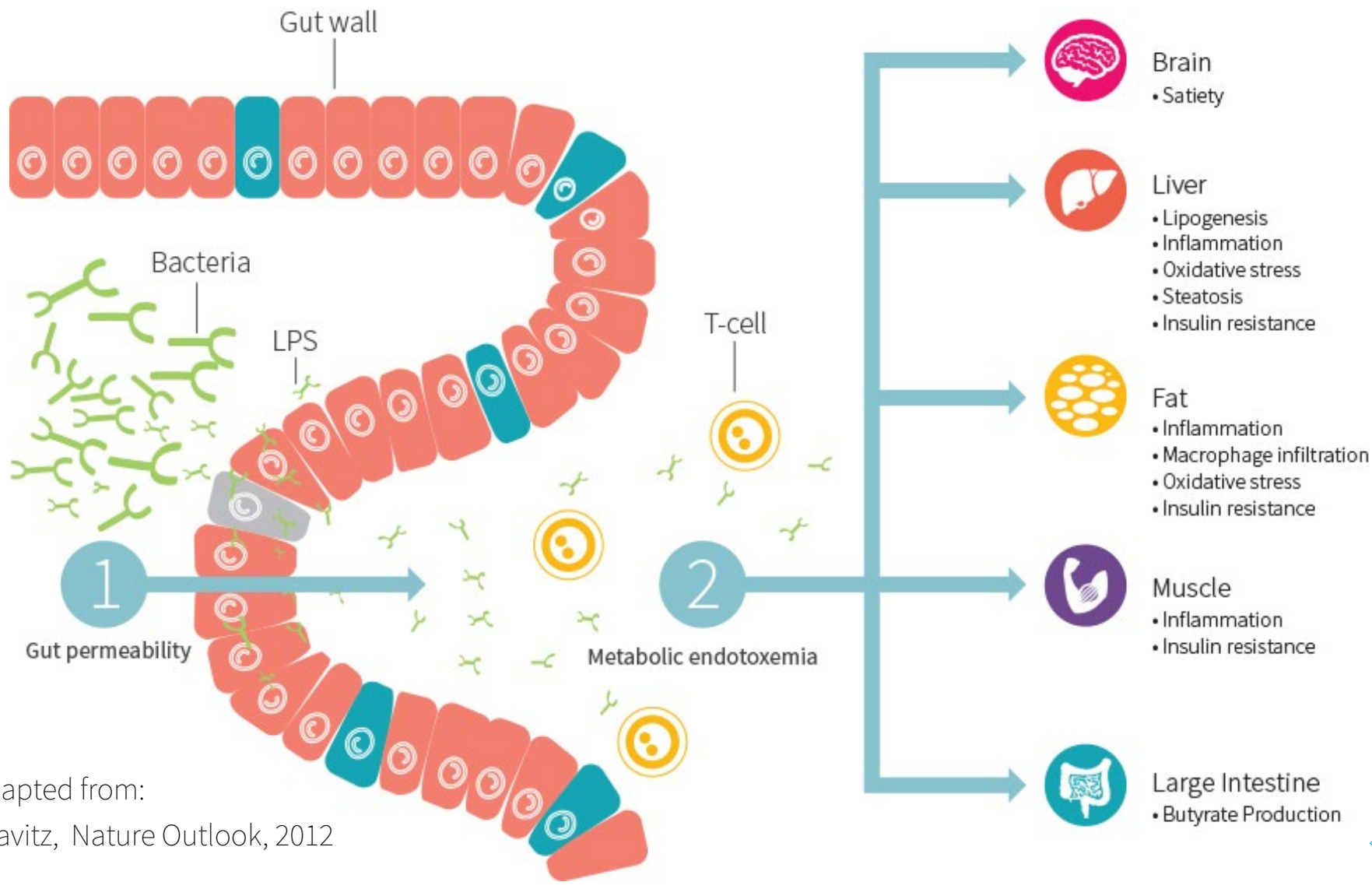
Who and what was studied?

In this study, 17 young, healthy participants of both genders were assigned to ingest either control or a yogurt containing *Lactobacillus casei* Shirota (LcS) for three weeks followed by one week of high-fat overfeeding.

The participants were at a normal weight (BMI of 18.5–24.9) and exercised at least three times per week for more than 30 minutes at a time. Their weight had been stable for at least six months, they exhibited good cardiovascular and metabolic health, and did not take any medication. Also, participants were required to not have used any probiotics for at least three months prior to the study and to not follow a vegan or vegetarian diet.

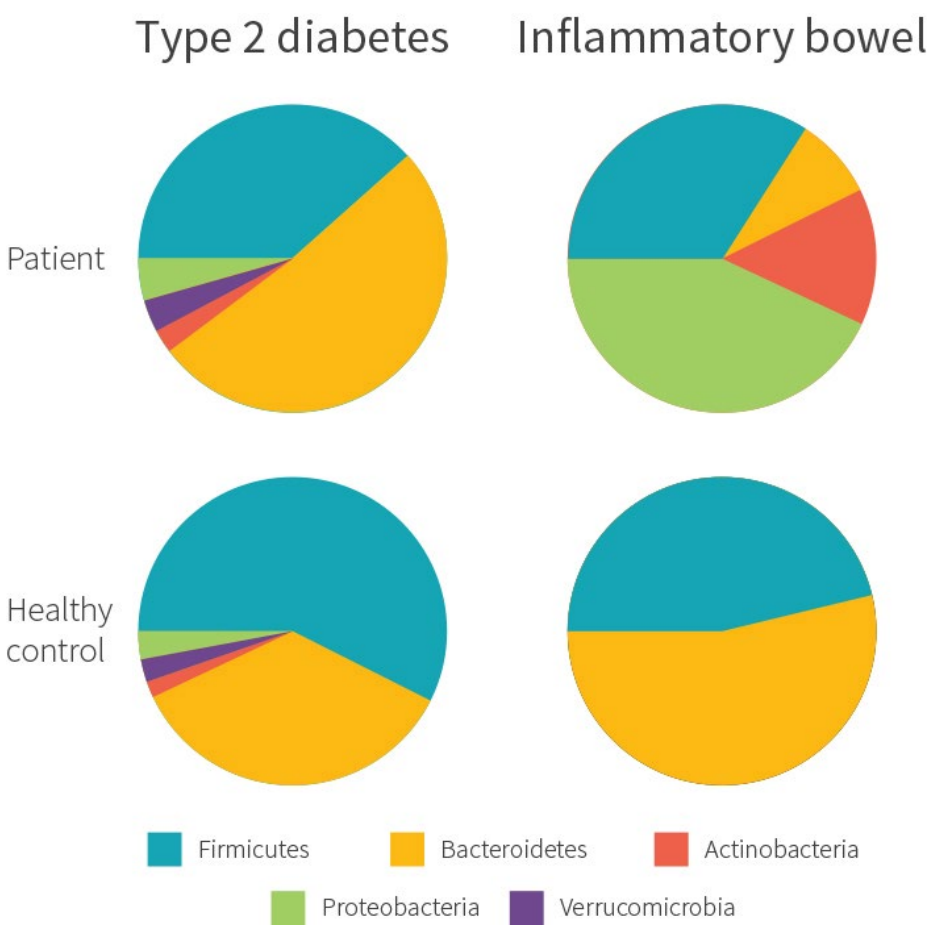
Before the onset of the intervention period, participants were weighed and had their fasting insulin and glucose measured. Next, study participants were randomly assigned to either the the probiotic group (n=8, seven males and one female), drinking a LcS-containing fermented skimmed

Figure 1: Mechanisms for gut bacteria influencing metabolic disorder



Adapted from:
Gravitz, Nature Outlook, 2012

Figure 2: Gut dysbiosis and chronic disease



milk product (Yakult Light) or the control group (n=9, seven males, two females), drinking nothing, not even a placebo.

The study itself consisted of three weeks of habitual food intake supplemented with 65 mL of the probiotic twice daily (only in the probiotic group), followed by one week of overfeeding. During the habitual food intake period, participants were required to keep detailed food logs, which included weighing food items, for three days per week. This was analyzed to estimate daily energy expenditure. After the habitual intake period of 21 days, the "real" part of the study began, i.e. assessing the effect of the probiotics on overfeeding-induced changes in glucose clearance. On day 22, all of the study participants were subjected to an oral glucose tolerance test (OGTT) and had fasting insulin, glucose and triglycerides measured. After that, the overfeeding phase began. Following one week of overfeeding, participants did another OGTT and fasting insulin/glucose/triglycerides sampling.

Overfeeding was intended to provide a 50% caloric surplus at a 65% fat intake for one week. Fat intake from the food logs was 90–100 grams a day during the habitual intake, while in the overfeeding period, fat intake was bumped up to 260–280 grams a day! Protein intake increased by approximately 20 grams a day, while carbohydrate intake decreased by approximately 80 grams a day.

Young, healthy people of normal weight either supplemented with a probiotic drink or were given nothing as a control for three weeks. They were all then given a 50% caloric surplus for a week. Glucose clearance, fasting insulin and glucose were measured before and after overfeeding.

What were the findings?

Weight gain and BMI

All of the study participants gained weight. The control group gained 0.6 kilograms, which was a statistically significant change, whereas the probiotic group gained 0.3 kilograms, which was not a significant change. The study did not report whether the between-group difference was significant, but given the sample size and variation, it most likely was not.

Fasting insulin and glucose

Fasting insulin was unchanged at 86 nmol/L in the control group and decreased slightly in the probiotic group, albeit not significantly so. Fasting triglycerides decreased in both groups (from 1.4 to 0.9–1.0 mmol/L). Fasting glucose increased from 5.3 to 5.6 mmol/L in the control group but remained steady at 5.8 mmol/l in the probiotic group.

OGTT results

The OGTT data consists of repeated measurements of insulin and glucose after ingestion of a glucose challenge. A common way to report this is as an area under the curve (AUC) which is, in essence, a summation of the data points. Overfeeding in the control group led to a significant increase in the glucose AUC by about 10%, with no change

Matsuda Insulin Sensitivity Index (ISI)

The Matsuda Insulin Sensitivity Index allows comparison between the results from an OGTT and those from clamp studies, which is considered the gold standard technique for directly measuring glucose clearance and insulin sensitivity.

The ISI is a common method used by researchers, with almost 3,000 articles citing the original article that describes the index. While modifications of ISI have been used for increased accuracy, neither ISI or the widely used HOMA-IR may correlate that well with other estimates of insulin sensitivity in certain populations.

in the probiotic group. A similar pattern could be observed for the insulin AUC data, but this was not significant, probably owing to greater variation in the insulin data.

Also, the mean OGTT insulin and glucose were combined with the fasting insulin and glucose in the calculation of the Matsuda Insulin Sensitivity Index (ISI). The ISI fell significantly from 5.3 to 3.9 (27%) in the control group only.

Probiotic supplementation prevented fasting glucose and glucose tolerance from increasing after overfeeding.

What does the study really tell us?

The study shows that the loss of glucose-clearing ability in (normal BMI, fairly young) humans induced by overfeeding can be prevented by a beverage containing LcS-containing probiotic—at least in the short term. The study was financially supported by the makers of Yakult, which doesn't necessarily mean anything with regards to the results, but should be noted.

One important problem with this study lies in the baseline fasting glucose levels. The fasting glucose in the control group was 5.3 +/- 0.2 (across nine participants) and in the probiotic group, it was 5.6 +/- 0.1 (across seven participants). However, looking at the numbers, the fasting glucose was higher in the probiotic group than in the control group before the intervention started. Whether this skews the data is hard to tell. On the one hand, this could mean that the subjects in the probiotic group were a little worse off to begin with (higher glucose) and therefore less likely to show a response to overfeeding (higher glucose again). On the other hand, it could also be argued that being slightly worse off, metabolically speaking, would reinforce the negative overfeeding effects as less metabolically fit people have poorer metabolic flexibility and adaptative resources. The first scenario may be the most likely, because the blood glucose values are within healthy ranges and the subjects are otherwise young and fairly fit. Therefore, a higher initial blood glucose level could make increases from overfeeding less likely to appear with overfeeding, thereby inflating the effects of the intervention.

The OGTT glucose and insulin curves definitely tell a story by themselves—overfeeding makes the control subjects much less efficient at removing glucose from the blood. But the fasting data and OGTT data were used to calculate Matsuda ISI and when we revisit those, we see once again that the baseline-calculated insulin sensitivity in the probiotic group was actually closer to the control overfed value than the control baseline value! Again, when insulin sensitivity is low, it may reduce the potential efficacy of an overfeeding period that should reduce insulin sensitivity, thereby helping to create an image that the intervention (probiotic administration) prevented a drop in insulin sensitivity.

Whenever a study can be shown to contain flaws that seemingly reinforce the effect of the intervention, and particularly when this flaw or error falls to the benefit of an entity providing financial support to the study, some red flags should

be raised. Although this probably is just an unfortunate randomization coincidence, the authors could have taken away any doubt by commenting on it, but they did not.

Furthermore, the authors actually had access to fasting insulin and glucose measurements from the pre-intervention period that could have been used to randomize the participants (i.e., distributing the subjects so that their average fasting insulin and glucose were comparable across groups), but this was not done. While this does not invalidate the findings of the study, it should raise some doubt about the magnitude of the reported effects.

Due to the pretreatment differences between the probiotic and control groups, there is a chance that the study effect sizes may have been exaggerated.

The big picture

Another study has shown that administration of probiotics for people with type II diabetes reduced fasting glucose and glycosylated hemoglobin (a marker of accumulated blood glucose levels), as well as several oxidative markers. The present study plays nicely into previous evidence, although the effects here may be exaggerated for reasons described earlier.

One limitation of this study was that the control group was not administered any kind of placebo, so it's hard to tell to what extent other components of the yogurt besides the bacteria may have affected the outcomes. Benefits might have come from the dairy, the probiotics, or both. Perhaps the researchers should have used a skim milk control if they wanted to isolate the effect of probiotics.

Sequencing gut microbiomes is all the rage in nutrition and metabolism science these days. Sequencing of ribosomal RNA can be used to identify which species are present in the GI tract and shotgun sequencing can be used to identify the genes present in the genomes, as an indirect way to describe the types of microorganisms, but also a way to get closer to knowing what metabolic pathways are influenced by which microbial profile. Also, assessment of SCFA content in stool and starch/fiber fermentation products can help elucidate how any observed metabolic changes in the study came about.

A recent study very elegantly used these techniques to show that saccharin produced long-lasting changes in the gut microbiota that resulted in impaired glucose tolerance

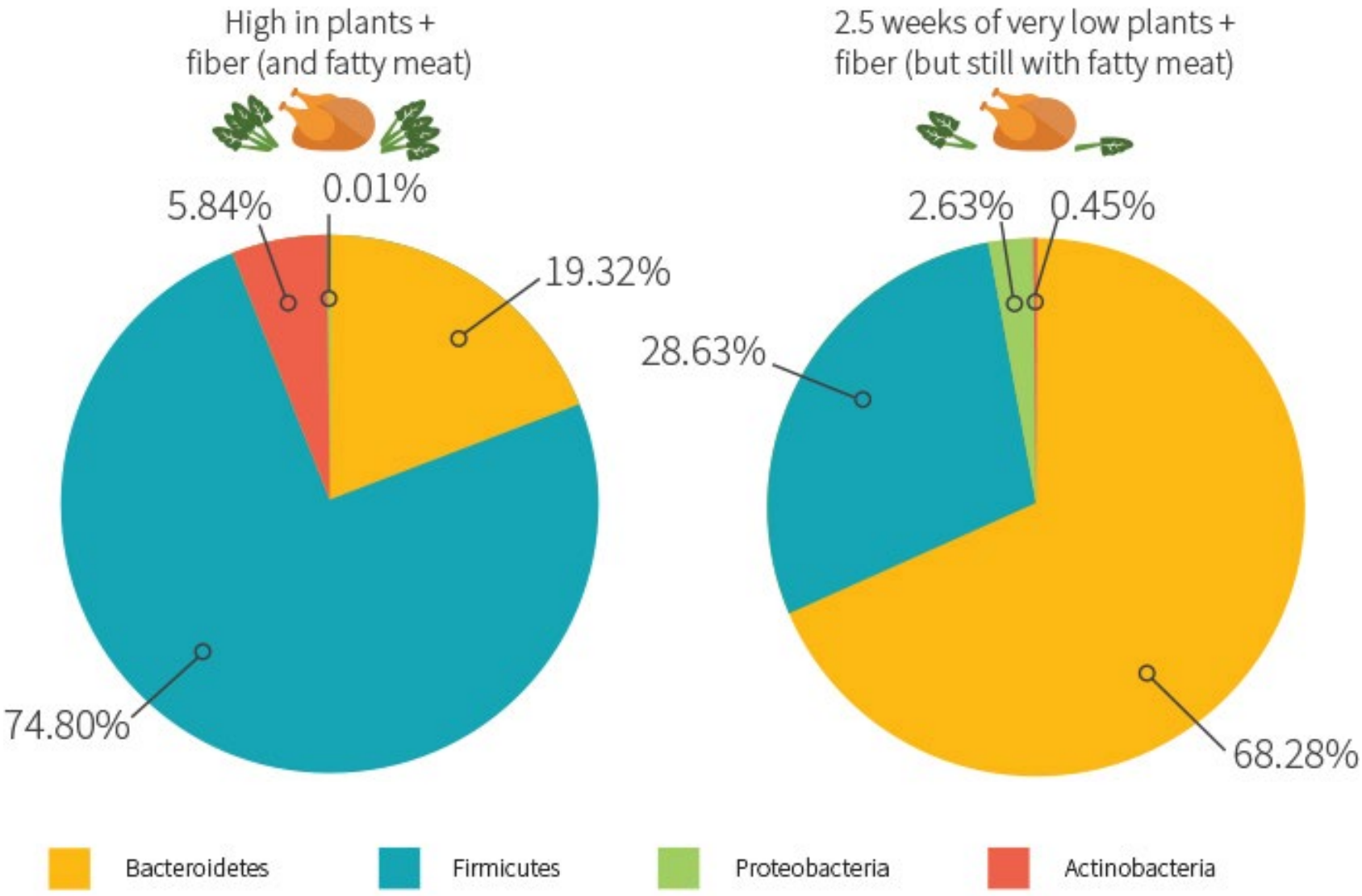
“One limitation of this study was that the control group was not administered any kind of placebo, so it's hard to tell to what extent other components of the yogurt besides the bacteria may have affected the outcomes.”

in mice, with weaker data in humans. This effect could be transferred to saccharin-naive animals through fecal transplants, in mice only. Without sequencing technology, it would have been impossible to claim that the intervention resulted in lasting changes in microbiota composition. A study like the current one would benefit immensely from these techniques (although trial costs could also skyrocket) because they would provide a peek into the composition and functional changes induced by the ingested probiotic. This would help determine if and to whom probiotics would be of benefit.

What's the real-world implication from this study? Although the study findings can't necessarily be attributed to the probiotic (because the control group didn't ingest anything at all), it's quite possible that the probiotic used in this study improved glucose tolerance after overfeeding. This matches well with several large cross-sectional studies (e.g., here, here and here) in which intake of cheese and fermented milk products is associated with lower all-cause and several disease-specific mortalities. Beneficial effects from probiotics might not necessitate supplementing directly with probiotics, however. Figure 3 shows a substantial change in gut microbiota after just 2-3 weeks of considerably altered plant intake, in an n=1 experiment from the founder of the Human Food Project (which focuses on the impact of microbiota on human health). While probiotics may introduce billions of bacteria to the gut, prebiotics from plant-eating may be able to feed quite a larger number and wider variety of beneficial bacteria that already colonize the intestine.

In the context of public health, the model of acute overfeeding isn't always a very realistic one. Some overweight individuals do not have large daily caloric surpluses, but rather sustained, small ones. Others go through cycles of overeating and undereating. One group of people who routinely expose themselves to acute overfeeding are fitness buffs during bulking phases intending to boost muscle

Figure 3: A shift in plant intake quickly changes gut bacteria



*n=1 experiment by founder of Human Food Project

growth. While the findings reported here could in principle extend to that type of situation and benefit insulin sensitivity, more research is needed to determine if that kind of overfeeding is even associated with impaired insulin sensitivity.

While this study did not confirm whether the probiotic supplement led to changes in the gut, other animal studies have demonstrated such an effect. Also, the nature of this study makes it difficult to generalize because it involved healthy subjects with large caloric surpluses over a short period of time, which isn't always how people gain weight.

Frequently asked questions?

What is the oral glucose tolerance test (OGTT)?

An OGTT measures your ability to control blood glucose after glucose ingestion. This involves removing glucose from the bloodstream, but it also involves suppressing endogenous glucose production by the liver. Both make important contributions to glucose control during an OGTT. It is normally performed through ingestion of 75 grams of glucose in the morning following an overnight fast. After the ingestion, blood can be sampled once at the two hour timepoint for glucose, or for research studies every 15 to 30 minutes for both glucose and insulin (in order to calculate indices such as ISI).

What does the "Shirota" mean with regards to Lactobacillus casei?

Lactobacillus casei is a species of bacteria with many subtypes, several of which are considered probiotics. One of the subtypes is the Shirota type described in this paper. Shirota lactobacilli were discovered/isolated by the Japanese scientist Minoru Shirota in 1930. Five years later, he commercialized his discovery by making it into one of the first probiotic fermented skimmed milk products, Yakult. There are other fermented milk/yogurt products in which the main probiotic microorganism is *Lactobacilli*, like Actimel.

What should I know?

Supplementation with a probiotic-fermented dairy beverage may prevent glucose impairment induced by overfeeding. However, because there were pretreatment differences between the studied groups, it is possible that the study's effects may have been exaggerated.

Also, the nature of the study limits its generalizability—people often gain weight as a result of small daily caloric surpluses, and not always as a result of periods of overfeeding. Future research in different overfeeding situations with different population types, possibly with a control group also ingesting a dairy beverage rather than nothing, can help confirm the present results. ◆

This study showed promise, yet had some important flaws. Does it make a strong enough case for these probiotics to be used in real-life situations? Discuss probiotics more at the Facebook ERD forum.

> "One group of people who routinely expose themselves to acute overfeeding are fitness buffs during bulking phases intending to boost muscle growth."

In closing...

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