Welcome, dear ladies and gentlemen, colleagues and family members. With this opportunity, I would like to share with you what I have been doing in this past four years, in our attempt to level up tempeh, that is by enhancing its anti-diarrheal potential using bacterial exopolysaccharides. To start, I would like to bring you along for a trip, although unfortunately only imaginary. Since the night is very cold today, how about we hop on a plane and travel to somewhere warmer, perhaps to my home country of Indonesia. When you travel to the global south, such as Indonesia, you might have heard the warning not to drink from the tap water. It is because in the tap water you can find bacteria known as intratoxigenic E. coli or ETAC. If these bacteria are ingested, they will enter your intestines causing diarrhea and thus ruining your trip. For most of us in this room, such diarrhea might be just a one-time incidence, but it's not hard to imagine that for people who live in these regions, diarrhea can be a constant health hazard. The way ETAC can cause diarrhea can be explained if we zoom into the surface of our intestines. Our intestinal surface is covered with carbohydrate molecules known as receptors. ETAC can bind to these receptors because they have a structure known as fimbriae on their cell surface. After binding to the receptors, ETAC will release entrotoxins, which can release... resulting in the release of water from the body. Now, how can we prevent this diarrhea from happening? Of course, we can use antibiotics to kill ETAC, but it's not a preventive method, and it can raise the emergence of antibiotic resistance bacteria. One approach is to prevent the addition of ETAC to intestinal surface in the first place, but to do this, we can use carbohydrate molecules that can act as receptor analogs, and these receptor analogs, they can bind to the fimbriae of ETAC, preventing them from adhering to intestinal surface, and allowing the cells to be safely flushed out from the body. Next, you might be wondering, where can we access this receptor analogs? Well, it's a good news that we decided to go to Indonesia for our imaginary trip, because one possible source of receptor analogs is a traditional and affordable Indonesian fermented food known as tempeh. The presence of receptor analogs in tempeh originates from the fungal fermentation of soybeans during tempeh production. This fermentation caused the breakdown of carbohydrates in soybeans into bioactive carbohydrates, which can act as receptor analogs. A different possible source of receptor analogs is a group of good bacteria known as lactic acid bacteria, or LAB. Some lactic acid bacteria can produce carbohydrates known as exopolysaccharides, or EPS, which can also act as receptor analogs. It is known that lactic acid bacteria are also present in tempeh. However, it is not yet known if the lactic acid bacteria in tempeh can produce EPS, and if they can produce EPS, whether said EPS can act as receptor analogs. This brought us an idea. What if we isolate EPS producing lactic acid bacteria from tempeh, and reintroduce it back into tempeh production so that it can dominate the bacterial population in tempeh and produce EPS? Will this co-fermentation result in leveled up tempeh with higher antidera potential to the presence of EPS in tempeh? And to answer these questions, we worked to first study EPS from a tempeh-associated lactic acid bacteria strains against ETEC addition, and second, to improve the anti-addition functionality of tempeh through supplementation with EPS producing lactic acid bacteria strains. The general approach to reach those aims was started by isolating EPS producing lactic acid bacteria strain from tempeh and characterizing their EPS structure as described in chapter 2 of my thesis. Next, we tested the strains capability to grow and produce EPS on soy substrate as described in chapter 3. We also investigated which structural feature of EPS plays an important role in the anti-addition by activity against ETEC, which is described in chapter 4. And lastly, we applied the strains in tempeh production to see whether it can influence the anti-addition by activity of the product, which is detailed in chapter 5. Now it is time to introduce you to the heroes of the story. I won't bore you with too much details on tempeh production, but long story short, we isolated four EPS producing lactic acid bacteria strains from tempeh production. Two strains, coded LMWA and LMWN, were isolated from water used to soak the soybeans, while the other two strains, coded PPTL and LCTL, were isolated from tempeh. Next, we grew each of the four strains in a liquid media that can stimulate EPS production to study their EPS structure. PPTL can produce two types of EPS at

roughly the same amount. These EPS were identified as dextran, which consists of glucose, and levan, which consists of fructose. LCTR, on the other hand, only produce highly branched dextran alongside insoluble EPS. In this project, we mostly only focus on the water-soluble dextran of LCTR, which was produced in the same amount as EPS from PPTL. LMWA and LMWN both produce dextran and levan. LMWA produce more levan, while LMWN produce more dextran. Interestingly, both strains have high EPS producing capability, producing ten times more EPS than the other two strains. Next, we wanted to know if the EPS can bind to e-text cells. To study that, we coated micro wells with EPS and exposed them to e-text cells. The unbound e-text cells were washed away, and if the EPS can bind to e-text cells, we can quantify them based on bacterial growth. We found that EPS from all the strains can bind to e-text, which gave us hope for their antiderial capability. Logically, we next wanted to know if the EPS can inhibit e-text addition to intestinal surface. To test that, we used intestinal mucosa from pig as a model. We coated the micro wells this time with mucosa, and at the same time, we mixed e-text cells with EPS and later added the mixture to the mucosa coating. If EPS can bind to e-... if EPS can inhibit e-text addition, there will be less e-text bound on the mucosa layer after the unbound cells are washed out, which can be monitored through bacterial growth. Unfortunately for us, none of the EPS showed the capability to inhibit e-text addition to intestinal mucosa. It was disappointing, but as they say, the show must go on. So, we tried to explore from another angle. We decided to focus whether the strains can produce EPS when grown on soy flour as substrate. This could be interesting since the production of EPS in solid fermentation using complex substrate is rarely reported. To do this, we inoculated the stress into soy dough, abbreviated as soy D, followed with incubation. After that, we studied the presence of lactic acid bacteria and repeated similar tests as before, such as EPS identification and anti-addition assay. We found our lactic acid bacteria strain can grow and dominate bacterial population in soy dough, with bacterial number around 100 times higher than lactic acid bacteria that are indigenously present in the substrate. Next, we found that only LCTR, LMWA, and LMWN can produce EPS. This EPS still showed the capability to bind to e-text cells, but more interestingly, the three strains showed the same EPS producing capability in soy dough just like in liquid media, indicating a robust EPS producing capability. Then, how about the anti-addition bioactivity? Soy dough derived EPS from PPTL and LCTR did not show anti-addition bioactivity just like our previous finding. However, we found that EPS from LMWA and LMWN can inhibit e-text addition to mucosa layer. This finding indicates that fermentation with the two strains can increase the functionality of soy product. However, we are still curious about the discrepancy of anti-addition bioactivity between EPS produced in liquid medium and soy dough. To get some answer, we analyzed the EPS using SPSEQ, which can give us information about the molecular weight of the EPS. To simplify, from SPSEQ, we can see the distribution of molecular weight as shown in these lines called chromatograms. These chromatograms showed that EPS produced in liquid medium, shown in straight lines, tend to have higher molecular weight but narrowly distributed, while EPS produced in soy dough, shown as dotted lines, have lower and more broadly distributed molecular weight. Based on that observation, we hypothesized that molecular weight might have influence on anti-addition bioactivity. To investigate that, we partially hydrolyzed high molecular weight EPS with enzyme and tested the anti-addition bioactivity of both the native high molecular weight EPS and the partially degraded ones. To our surprise, the high molecular weight EPS did not show anti-addition bioactivity while the partially degraded ones did. This indicated there is an optimum molecular weight range that can trigger anti-addition bioactivity, and high molecular weight might be detrimental to state bioactivity. Nevertheless, we now have two prime lactic acid bacteria strain candidates for application in type A production, LMWA and LMWN. Both strains can produce EPS in considerable amount when grown on solid substrate, and their EPS can exert anti-addition bioactivity against attack at certain molecular weight. And so that's what we did. We added each of the strain into tempeh fermentation. And here's what the product looks like. We found that supplementation with either strain did not affect fermentation and visual

appearance, other than slightly inhibited fungal growth at 24 hours. Moreover, bacteria profile of tempeh produced without lactic acid bacteria supplementation is dominated by spoilous bacteria known as bacillus, while the supplemented tempeh is dominated by our strains. This is a good start because it shows that our strains can persist in tempeh production, which is a microbiology-key diverse environment. So how about the EPS? Are they present in tempeh? We found that LM1 was not present in lactic acid bacteria supplemented tempeh. However, the extern was present in the supplemented tempeh, but was absent from the unsupplemented tempeh, indicating that EPS was produced by the supplemented strains. For the anti-addition activity, we found that tempeh extract can inhibit attack addition to mucosa layer, which was to be expected. However, our biggest surprise was that extract from tempeh supplemented with the lactic acid bacteria strains showed stronger inhibition up to three times stronger than unsupplemented tempeh. We also confirmed that this stronger bioactivity was driven by the presence of dextran in the tempeh. In a nutshell, what is our main finding? Well, as explained by this cartoon, if we add EPS producing lactic acid bacteria along with fermentation starter in tempeh production, we can get tempeh that contains that lactic acid bacteria strain. That strain can produce EPS during fermentation process, giving us our leveled-up tempeh. If we compare that with normal tempeh extract, normal tempeh can block attack addition to mucosa layer, but not completely. However, our leveled-up tempeh is more effective in inhibiting state addition. And that brings us to the main conclusions of my project. The first conclusion is that first, the presence of EPS, particularly dextran, in tempeh can improve anti-addition bioactivity of tempeh against attack. And second is that lactic acid bacteria supplementation can be a viable strategy to produce EPS and rich tempeh. But before I end this presentation, I would like to give this main take-home message is that we found that bacteria are an inherent part of tempeh production that can contribute to many aspects, such as health-related aspects, particularly anti-diurnal potential. And therefore, industrial standards need to be ready to adapt in order to accommodate the development of leveled-up tempeh using bacteria supplementation. And now I would like to briefly describe the next agenda. After this, I will defend my thesis and proposition for 45 minutes, which will be followed with 15 minutes deliberation by the committee. And after that, I hope that I can sign and receive my PhD degree so that I have a good excuse to invite you all for a dinner party at H41 at 6 p.m. And thank you for your attention. Please be seated. I hereby open this ceremony convened by the Academic Board of Wageningen University in which Theodor's Eco-Pramodito is offered the opportunity of defending a thesis with propositions entitled, Leveling Up Tempeh, Enhancing Anti-Diarrheal Potential of Fermented Soy with Bacterial Exo-Police Secarides. The defense will take place before an examining committee appointed by the Academic Board as a prerequisite for conferring the degree of Doctor. Good morning. I would like to welcome you all to this graduation ceremony. My name is Monique van Ursh. I'm Professor of Virology and representing the Academic Board and Director of Magnificus today. I call on the first examiner, Professor Skaustra, Professor in Microbial Ecology here in Wageningen. The floor is yours. Thank you, Madame Rexxar. Respected candidates, I applaud you on the completion of your thesis and also on the very nice cover that you made. It looks really beautiful. And I have some questions for you. First of all, in Chapter 2, you described that, well, you isolated four strains that produced the exo-polysaccharides. So, the number four, did you make a large effort to get four, or could you easily have gotten more than four? Hi, yes, the opponent. Thank you very much for your kind words and also for your comments on this, on the proper. Yes, well, four doesn't come from some defined inspiration. Actually, we started from around 26 strains that we isolated, but those are 26 strains that we noticed are capable of producing exo-polysaccharides. But after initial identification screening using API-based approach, we found that most of these 26 strains, they share the same species. And therefore, we narrowed down to initially six largest groups, but we realized that from that six, two didn't produce EPS in a consistent manner. So, that's why we windled it down to four. Okay. Thank you. And you used a certain way of processing

tempeh. You described it in your Chapter 2. If you would have used another way to make tempeh, would you have gotten different strains, you think? That is an interesting question indeed. And for certain that getting different strain, yes, but getting different species, that's another question, because I think in general, tempeh bacterial population is very diverse, but at the same time, the same bacterial species tend to be present in most consistently across tempeh fermentation. But that also depends by what variation of production process that you want to explore. Yeah, so indeed, so on this variation in processing practice, you are from Indonesia, so I'm sure there's lots of variation in the traditional processing. Indeed. So, how does this method in Chapter 2 relate to the traditional processing? Yeah, the method that is described in Chapter 2 is pretty much the modern version of the production process, because we even explored, like, for example, WA. It's from a tempeh that is the soaking process in fast acidification instead of natural acidification, so that represents something that's more often used in the industry. But, for example, if I can put it back in the context of production in Asia, I'm particularly personally interested, for example, in a traditional method such as if the tempeh are, for example, wrapped in bananalis, probably we will get more. And sorry, if I may interrupt. Yes, please. Do you think that there's natural variation, let's say, for the traditional processing, and you also say that tempeh can be used as an oral therapy? Do you think that there is variation also in how much EPS there is produced, based on what type of traditional processing is used? Yes, there can be variation in how it is produced. But I think what we also consider is that, of course, tempeh can also be a tarar-based product in the sense that maybe in a processor, because they already use the same starter again and again in an environment that is more or less confined to a certain place, maybe we can get more replicability with certain degree of variation, at least in that limited producer. And could you ask one of your paradigms to read Proposition 2, please? Paradigms? Could you please read Proposition 2? The role of bacteria as an inherent part of tempeh production is undervalued by the industry. Thank you. Do you think this is also undervalued by traditional processors? Yes, it's still undervalued because the main paradigm of tempeh is, of course, fungi, the fungal starter, and soybeans. But the emphasis on the role of bacteria is now only starting to get explored more and more. And then you explained that this production of EPS is a survival mechanism for the bacteria. Yeah. So during this fermentation, what are the bacteria surviving from? So what specifically sparks the EPS production? You can try to imagine that in a tempeh production, the bacteria is, at least the lactic acid bacteria, they're facing a diverse group of other microorganisms that could be used, also the fungi itself and also the other microorganisms. So you think it's a species competition? Yes, it's a species competition. So this EPS mechanism could be one of the ways that they can hoard the resource for themselves. And then in Chapter 5, you described that these adjuncts displace other lactic acid bacteria. Would that also be then connecting to your answer that you just gave? So why would those adjuncts displace the existing lactic acid bacteria? Does it also have to do with their survival strategies? Yes, indeed. Because, of course, by true supplementation, we basically added this one specific group of bacteria in a larger amount. And for that, they're much more easily dominated in the population because they have power in quantity. Madame Rechter, do you have time for more questions? You for sure have. Thank you. So now this EPS is supposedly inhibiting this E.

coli that is causing disease. So let's say you would now have the same EPS being produced in this way that you're suggesting. What would that do to the E. coli? Do you think the E. coli would adapt to this and would it become resistant? Yeah, I could not remove the possibility of resistance. But I'm certain that the resistance will be more in the long run, certainly, compared to antibiotics. Because at least through the use of bactericidal approach, we will trigger more natural selection. But with this, the E. coli will be safely flushed out from the body. So basically, we are just kind of like inhibiting them from getting a resource-risk environment to another environment where they can still strive. So there is less of

environmental pressure. What do you exactly mean by natural selection here? Natural selection is because if you use bactericidal, that means you will more or less kill most of the population. But you will leave a proportion that is resistant to that bactericidal agent. Okay, so that could be – yeah, but do you think for EPS that it's not such a big deal? Yeah, the chance will be much lower compared to conventional approach like antibiotics. Okay, well, thank you. Thank you, Madame Rector. Okay, thank you very much. The floor is now for the second seminar. Professor Waverst, Professor of Food Chemistry, Martin Luther, Universität Haller Wittenberg in Germany. The floor is yours. Respected candidate, I congratulate you on your interesting results on the properties of exopolysaccharides. I also found it very interesting and enjoyed reading that you investigated the structure more closely, as this is sometimes a gap in literature and you successfully filled it. So, I have some questions. You found it especially interesting from my point of view, exopolysaccharides from leukonostociturium, where you saw that it produces insoluble glucan, and you also tried to separate this. Do you think it is possible that there is more than one glucan in this mixture produced by these organisms, and what would be a number of different glucans produced by different enzymes you would expect? Hi, yes, Tim Oppenent. Thank you very much for the canvas, and I feel glad that you find my thesis enjoyable to read. And yes, that's indeed an interesting question about the property of leukonostociturium. From the result that we have so far, at least, what I can say with certainty is that there are two major groups of glucan. One is the dextran-like highly branched glucan, and the other one is the insoluble glucan itself, because at least from all our evidence seems to point towards the evidence towards those two major groups. So, you think they are produced by different enzymes or by one enzyme? It's likely they are produced by multiple enzymes, because, of course, we did a simple analysis on the diversity of glucosyltransferase enzymes that are present in the strain, and we found quite a diverse amount of enzymes there. But yeah, unfortunately, we didn't identify it to the more accurate level. Okay, okay.

So far, you described the dextran as a highly branched molecule, and based on your structural analysis, you got many hints in different directions, and you concluded that it's likely O3 branched or highly branched. Do you have hints that the position of branching or the overall structure could be different from the ones you've also characterized, for example, from the mesenteroidus strains? Do you mean by position, you mean like a distribution of the branch? Yeah, where the glucose units are attached to the 1,6-linked backbone. So, you described them to be linked to the position O3, likely.

Do you think it could also be linked unaware? Yeah, it's also most likely that these branches are linked with alpha-1 for a position, because we noticed that if we treat them with amylo-glucosidase, we also see a further degradation of this life offer of the peaks that we detect in HPAEC. Yeah, and I mean, do you have also some hints from your PGC or HPLC-MS analysis? Do you think there might be other compounds in there? No, at least we tried to explore the possibilities of organic acids present in the EPS structure, but at least when we are targeting for, like, acetyl structure, we couldn't find any. So, yeah, at least it seems the direction seems to point forward to polysaccharide structure, but, yeah, it's just more complex in the structure-wise. What kind of analysis would you conduct if you still had, let's say, half a year time or so to characterize it more closer? Okay, definitely linkage analysis.

And probably we also would like to explore more on other types of NMR that can give us more, allow us to shed more information about the fine structure of the... What would you expect to see from that? So, what kind of NMR experiments would you suggest to use? Okay, for NMR, at least the first that comes up to my mind is the... I forgot the first part, but it's a spinning magic angle NMR, which seems to be very commonly used in the characterization of insoluble polysaccharide, insoluble glucan. But, yeah, to be honest, I also haven't explored much on that

angle as well. And I mean for the soluble ones, for the water soluble ones, could you imagine a solution state NMR technique? Perhaps that for the solution state NMR techniques, it's doable for the soluble fraction, but, I mean, we've already tried for the insoluble EPS LCTR and it just gives broadening in the peaks, so, yeah, that's pretty much difficult to apply. Yes, the high viscosity won't help with that, so linkage analysis may help.

So, for the insoluble ones, you also described that it's possible that you have a mixture of one-six linked and one-three linked polymers, or that you have a copolymer.

How could you distinguish that? Okay, the simplest way to distinguish it is, of course, by at least from what I can imagine would be through an enzyme-based approach, because assuming that if it's a copolymer that is interacted with another insoluble structure, then it will give a different profile after degradation compared if it's like a mixed linkage. A different profile if you apply which analysis, or would you analyze it after the digestion? I would use, of course, with dextrose with partial hydrolysis, most likely, partial hydrolysis, and then analyze the structure that's released, and then compare that, not compare, I mean, try to make sense of what resource that we get. Yeah, with which technique, I mean, sorry, which chromatographic technique would you use? Yes, at least my hypothesis is that if the second scenario that is with the two copolymers that are interacting together, there's a very high likeliness that there's supposed to be ideally more linear, because if it's highly branched, then it will be more difficult for them to form hydrolysis. So, theoretically, it's supposed to give less branched oligosaccharides that could be seen with this analysis. So, but what's the problem with the 1-3 linked if we want to hydrolyze them with the enzymes? Yes, so the 1-3 linked is very much the cause of insolubility in this kind of glaucon structure, so most likely that the enzymes that we have, they have difficulty of accessing the structure that is insoluble. Yes, that's a big problem. So, you also found in Chapter 2, you analyzed the contents of glucose and fructose after chemical and after enzymatic hydrolysis, and you find similar amounts of glucose and fructose, and as you're using a dextranase, which is also producing isomaltose, do you think this makes sense, or is there some limitation to each approach, what amounts of glucose, for example, you receive from a polymer? Sorry, could you please repeat the question? Yes, the question is aimed at, could something happen during acid hydrolysis, which limits the amount of glucose you find? I mean, with the enzymes you get isomaltose and glucose, this limits your glucose amount. Is there also something in acid hydrolysis which could limit the glucose amount you find? The most likely for limitation in acid hydrolysis would be if the polysaccharide is insoluble, because it can be difficult to degrade with methanolysis. But, at least in the case of leuconose mesentroidus, it's pretty much easily degradable.

So, at least for now, we didn't see much, what is it, reason to be skeptical of the research that we got from the acid hydrolysis. Yeah, but what happens if you, for example, cook glucose, pure glucose in acid? Yeah, it can undergo through reaction, but at least from what I've seen so far, dextran is also in the same glucose.

Of course, it can be degraded, but the method that we use is already, where we try to optimize as much as possible to minimize such for the degradation. Yes, of course, but you probably will always have some degradation.

This is unavoidable, but could you also combine the dextranase with another enzyme to get a higher amount of glucose, which is not prone to degradation under the conditions? Yes, theoretically, it would be possible, but I try to find more information about enzymes that can specifically, for example, degrade isomaltose so that we can get total degradation of the glucose. Because, yeah, for example, it can make our calculation more difficult if we have life over isomaltose, isomaltotriose, and some other DP. So, yeah, that's a good start by exploring more

enzyme, but at least from the result that we have so far, yeah, it's a very comforting, at least in the case of liquid isomatroids, it's pretty much consistent between the two methods. Yes, I mean, I would agree. But it's starting to get challenging. Yes, but you've got the important information, I would absolutely agree.

And maybe one last question. You used HPSCCRI with polarland standards to estimate or to determine, you wrote, to determine the molecular weight distribution. How much is it a determination rather than an estimation? So how exactly can you determine the molecular weight with that? Yes, that's a very good question indeed.

And, yeah, of course, estimation is the, let's say, the most accurate way to describe it. But at least from what I've seen so far, and also I've read from literature, when we are still talking about the molecular weight in around somewhere around the pants of kilodalton, it's still pretty much comparable. But I agree that once we go much higher, I think from my observation, it seems to be higher than somewhere around 200 to 300 kilodalton, then it will start to give some deviation between, for example, if you use standards between what the manufacturer says and also what our method sees. But at least for our project, since we mostly also focus on the functionality as well, the information that we get should be sufficient. But, yeah, I think using, for example, like a mouse as an approach would be a very much favorable choice for that. Yeah, but I agree that you extract the important information. The problem about the mouse is you need the injected mouse most of the time, and this is difficult for samples from natural sources and also from food.

So, thank you. Thank you very much. And I would like to give the floor to Dr. Raubos from the DSM family. The floor is yours. Thank you.

With respect to candidates, congratulations with a nice thesis. It looks nice, but also the content I think I read with great pleasure. It was really fun to see how the work on Tempe continues after I did a long time ago my PhD on Tempe. As I'm not working with Tempe anymore, we're working now in an industry and working with yogurt. So my first question to you will also be in that direction. As you know, also in yogurt production, we use a lot of lactic acid bacteria.

Also, EPS is very relevant in yogurt production, but mainly aimed for texture. So, can you illustrate a bit about how your insight from this research in a health perspective is that translatable also to the yogurt industry? Hi, my name is Tim Oponit. Thank you very much for the nice comments and also thank you very much for being a great inspiration for this whole project. Yes, that's very interesting indeed.

With the yogurt industry, because at least for the EPS that we mostly explore, it's stimulated by the presence of sucrose in the substrate. I think with yogurt, at least if we stick to the basic yogurt production, I don't think we will encounter much sucrose unless we supplement the fermentation in a high amount. Although, full disclosure, I haven't explored so much in a milk-based medium, whether it will still produce EPS in a similar nature or not, but let's just assume that it does not. But I think what will be more interesting, in my opinion, is the application in the more vegan side of things, especially if you want to explore more like a soy milk-based yogurt, because it's already an ideal substrate for the EPS production. And maybe it's also tubers with one stone. It could be a texturizer and also can bring some extra functionality to the product. To go back a little bit about your statement in yogurt production, because EPS is really relevant in yogurt, so there is a lot of EPS, although it's maybe another type. But do you see linkets?

Because some specific types of lactic acid material also in yogurt production can produce dextran, although it's not the general ones. But how do you see this? Yeah,

for yogurt production, of course, it's very much applicable.

It's lactic acid bacteria, so by that sense, it's also easily translatable in yogurt production. But as you already mentioned, I think with yogurt production, we're already spoiled with so many alternatives and so many choices, because of the lactic acid bacteria produce EPSs in, at least in the milk-based substrate. We already have so many reports about that. So the question is, what do we still need to push further? Or maybe the idea is that if we can try to characterize on what EPS is already present by default in yogurt, and then see if this EPS also exhibit anti-addition by activity, because it could be a good idea of a product. But before people travel to somewhere, they can try to acclimatize their gut microbiota with yogurt products, because it's more, of course, especially in Europe, it's more easily accessible. What's your expectation about that?

Because that's the field I'm aiming for. How can we have this nutritional benefit of anti-adhesion?

Because on a normal yogurt, it's not claimed like it can work for yogurt, but not typically on this anti-adhesion. Are there possibilities?

Do you see possibilities to do such work or to implement that in yogurt? Yes, indeed.

There is a possibility, but the possibility is there, but I think sky is the limit.

And as I mentioned, because with yogurt, it's very much already explored. No, I mean the presence of EPS in yogurt is already explored, but I think no one has, or at least still a few, steers this research direction more into an anti-diarrheal approach.

Yeah, it's a good idea to explore further. Good, thanks.

I want to go into another part of your thesis. Like in your general discussion, you describe also bacteriocene production, especially produced by the lepticus bacteria that you isolated from the soaking water. And then you say also, okay, they can inhibit the growth of bacillus precis, but then in chapter five, you see that there's hardly any bacillus anymore in the product, but also it's related to the lefon production, but you don't see in the tempe. And then you say, okay, maybe the bacillus will break down, so you cannot form the lefon anymore.

So it's a little bit going into different directions. Can you explain a bit better? Yes, indeed I can understand the contradictory elements of it, but I would like to emphasize that the part that we still haven't explored much is the dynamics of the fermentation, because we only monitor the tempe at the 24 hours and 48 hours. So there's two possibilities.

One could be that maybe in the early part of the fermentation, there's more competition that makes it not ideal for the strain to produce lefon, or maybe because it faces more competition. And also another possibility is that we also don't know whether, for example, if the adjuncts strain themselves, maybe they also need this lefon for their own benefit, especially in this competitive environment, so that's why they keep on degrading. But at least if we go back to the scope of bacillus, it's a possibility that maybe in the early part of fermentation where the bacillus was not yet fully suppressed yet, the environment was not ideal for the strain to produce lefon. Okay, yeah, and I also want to connect it a bit to the research I did a long time ago, so I also explored some inhibition activity about certain bacillus species, but then I observed it just in the tempe and not in the soaked beans. And as it has to do with the same bacterial scene, then you will also expect, I think it also in the soaked water, because bacillus will most probably

also be there or not. But do you have from your research any more insights, because I define it just to be a protein compound that can kill the bacillus, but for your new insights, can you, do you have any more clue what type of compound this can be that is specific for tempe and not found? In the cooked and soaked soybeans? You mean the possible compounds that inhibit the presence of bacillus? Yeah, yeah. Yeah, as I mentioned in my thesis, of course, the prime suspect falls into bacterial scenes, which also falls into a protein group. But then do you expect them in the soaked beans as well? That's a good question. Yeah, because there is the difference, what I found in my research, and I, yeah. Well, I cannot say for certain, because, and also, yeah, but if it's an environment that's where the strain faces competition, I think bacterial scene is something that the strain really wants to produce. But another possibility, especially if we add adjunct strains, is that other than the presence of certain compounds, it will also be the fact that there is a lack of compounds, because, you know, these adjunct strains, they consume at least they have more quantity to consume the nutrition that kind of suppress the other microorganism from growing. That's another possibility. But if we want to explore the presence of compounds, yeah, we have to explore further on that possibility. Although there is also some reports that say that exopolysaccharides also have some antibacterial inhibition capability. I don't want to make a bold claim yet, but that's a good angle that we can try to explore. Yeah, and to have a little bit more on the microbial composition, because in the tempeh you have quite a rich microbial composition, but you do like steps like cooking in between before you produce the tempeh. And in the soaking you create the lactic acid, also there is quite some replacement. And then you try to work as sterile as possible in the next step. So I'm always wondering where this complex composition comes from. Is it all from the beans? But then you expect more or less the same composition in the soaked beans as in the tempeh, or, yeah. Yeah, that's actually a very deep question. I think that's the holy grail of so many tempeh researchers right now are currently looking. At least the currently accepted school of thought is that it likely comes from the tap water. But, of course, another question comes. Is it from the tap water itself, or is indigitously present in the soybeans and then proliferate during the soaking step? But, yeah, the common consensus right now is, first, it's from the tap water. And also another hypothesis that now it also starts to pick up pace is that there is a possibility that the fungal starter, at least in the context of the commercial production, the fungal starter that people use often already comes in symbiosis with certain groups of microorganisms. Those are the two schools of thought that are most common among this topic currently. But in your lab you work with a quite defined culture, and I think also worked with quite clean water, or used even sterilized water. I don't know if you did. So, yeah, from traditional production I see all the steps. But like when you make the tempeh cleanest possible in the lab, we also serve some great diversity in strains. Yeah.

At least in the lab scale, my hypothesis is that if we stick to the tap water or the soaking water hypothesis, it's most likely that there is an abundant of microorganisms during the soaking step. And that the cooking process, although it kills most bacteria, but because of the high number, there is still quite a population that is still left behind. Because you can imagine that 0.1 percent of, for example, like hundreds of billion, that means still quite a significant amount that could still be present. That's at least my idea of what could possibly explain it. Do I have time for one more quick note? Unfortunately not.

Thank you very much. I'd like to give the floor to Dr. Kieres from NOGI Technologies in Delft. Thank you, Madam Chair.

A respected candidate. First, I would like to compliment you with your thesis. It's almost as thick as a brick of tempeh. And honestly, also after reading it, I found it quite nutritious. I'm happy that you also flavored it with some nice illustrations and photo, but because in my opinion, that's also what tempeh needs.

I also want to stress that I've been in the tempeh field for quite a while, quite a long time ago, and I'm really nice to see how you built on the earlier work done. And also to see that these ancient fermentations can be leveled up in your words to become even more functional for the future and to have more benefits than already there. So today's a special moment, and I would really like to dive a little bit deeper with you in your thesis. And the first question links to the isolation of the four strains that was earlier mentioned. I would like to know what is the relative abundance of these typical EPS-producing strains in the natural tempeh? Hi, I'm Samuel Pona. Thank you very much for kind words. So, yeah, basically, it's a very nice compliment for me as a nation. That means I make good metaphoric tempeh. For the relative abundance of this microorganism in tempeh itself, we did not analyze a breakdown of the distribution of species, but if you allow me some guessing, I can imagine that the amount would be not so abundant. Because, yeah, or else if it's too abundant, it's easy to imagine that there should be some textural effect of these strains of presence in tempeh, but we don't see that. And second is that lactic acid bacteria, or at least leuconazole, is also not to be like the most abundant group of microorganisms in tempeh, at least from what I've already reported, because gamma-proteobacteria and vassals are more common than these. Basically, we are kind of like fishing specific fish in a more wider array of other fish, so it's not the most common population. Thank you. You already mentioned that these four strains came from a wider selection of over 20 different strains, so in that way that's already dealt with. I was just wondering whether there would be an alternative to supplementing tempeh in the process with starter cultures or edging cultures. Could you use also substrates that these specific EPS-producing strains could use in order to be growing a little bit more? Yes, that's a very nice idea indeed. And if I'm allowed to be wild with my idea as well, I also have this thought that maybe we can also supplement tempeh production with sucrose, because that's a substrate that is needed for this EPS-producing. So without supplementing specific microorganisms, we can just add more sucrose, because it can trigger EPS production that we can create an environment that is more suitable for these EPS producers to produce EPS. But of course, we have to think about the effect of the flavor, because we want to add sugar to have extra sugar in the tempeh. And second is that we also need to know what the other microorganisms, particularly the fungi, will do with this substrate as well. Thank you for your answer.

When we look back to the original way of producing tempeh in Indonesia, for instance, you see the traditional use of accelerated acidification through backsloping, I think it's called, in the past. So how about using starter cultures?

So this is also maybe an industrial economic question.

Using starter cultures requires typically to buy new cultures when you produce a new batch of tempeh. Would you think that the strains that you have selected in Chapter 5 to produce the EPS-rich tempeh would be eligible to use basically to recycle them? Pardon me, but by recycling, you mean like a backslope approach, or use it for development of starter culture? Do you need to have new starter culture with every production, or could you use, for instance, an earlier batch of tempeh to inoculate the next batch of tempeh? At least from the microbiome research that we saw how the bacteria dominate the tempeh population, it has a potential to be used for a backslope approach. But yeah, I cannot guarantee you that in a 2 or 3 or even 10 or 20 states of production, whether it will stay constant or consistent. So at least I personally prefer the approach of developing a starter culture that we can add as an adjunct in tempeh fermentation. I think there's also a downside maybe to using the starter cultures, and I'd like to have your opinion on this, because I saw in one of the tables, 5.2 in Chapter 5, that the water soluble fraction of the tempeh, which is not inoculated with your strains, is actually quite significantly higher than the tempeh that has been used with the adjunct cultures. So to me, there would be a nutritional benefit over the non-inoculated tempeh.

So could you elaborate on that? Yes.

Of course, the risk that we always face whenever we treat or interfere with the natural sponge experimentation is basically we create an artificial environment that makes it less diverse, or at least can impact the nutritional capability, sorry, the availability of the product. And that's something that maybe we have to explore further regarding this characteristic. But the idea is that through the use of an extra fermentation culture is that we can make a more specified product to enrich the availability of the product that is available on the market. So yeah, either it could be a trade-off, or it could be an enrichment of the choices that we can have. For example, if we can have, of course, the default tempeh, but we can also have a more functional tempeh, because I believe that people who are preparing to, or at least, because nutritional availability is still there, maybe it's not so high, but also keep in mind that tempeh is also not just consumed as tempeh in Indonesia, so it accompanies other kind of foods. So it's still a source of nutrition, maybe could be less optimal than the default tempeh, but at least that lack of nutrition can still be easily covered by other strategies that's not limited to the tempeh itself. Thank you.

That brings me to the next question, which deals actually with the actual value that you bring by increasing this EPS production and the potential antiderital effects, because indeed it is a trade-off, in my opinion. So we definitely need to see what is the value of making these extra efforts in bringing tempeh to the next level. So by doing that, you use various screening essays for detecting the potential to interfere of tempeh and tempeh fractions and EPS, rich fractions, to interfere with the adhesion of E.

coli, enterotoxic E. coli. So you gave a nice overview in the first table in chapter one of different essays, so could you elaborate a little bit and motivate why you choose the essays that you used, and not other essays that are also described in that table? You allow me to check first?

Is it the one with the plate-based method? It's the table 1.3, where you list a couple of methods used to measure anti-adhesion by activity, and it ranges from, of course, the cumbersome in vivo testing towards the more simplified methods that basically are the category that you chose to use in your work. The method that we ended up choosing is because we would like to have a high-throughput approach that's also less time-consuming, because, especially in the majority part of my research, we have more exploratory approach, so that's why we need to test so many tempeh samples with different parameters. So that's why we found that this method is the most ideal for our high-throughput approach, because we also explored using- we actually also done a trial with other methods that I described in this table, and some methods are first either too cumbersome, because we have to, for example, we have to follow microplate assay with screening on plates, or they don't show a consistent-or at least it's not robust that we don't get a good replicability. And from all the methods that we have tried, the mucin-based analysis using microplate approach is the one that ensures high-throughput analysis, replicability, and also a good simulation of what's present in the gut. Can you elaborate on the letter, the good simulation of the presence in the gut? How sure are you about that? So, basically, with ETEC infection, it starts with the ETEC accessing the mucus layer before it can gain access to the intestinal surface.

Of course, we can also-the thing is that we also need to- Please be seated. I hereby reopen this meeting. The Academic Board of Wageningen University, represented by the Deputy Director Magnificus and seven committee members appointed by that academic board, have noted the contents of a thesis entitled, Leveling up Tempe, enhancing anti-dearrheal potential of fermented soil with bacterial exopolysaccharides with propositions, having heard the defense of that thesis, has decided to confer the degree of Doctor on Theodoros A.

Golramuduti, born in Jakarta, Indonesia, on November 13, 1988, and to grant to this person all rights and privileges ensuing from their doctorate by law and custom. The Academic Board assumes that you accept your duty as a scientist to execute your future research ethically and with due diligence according to the Netherlands Code of Conductful Research Integrity. I now invite the promoter, Professor Scholz, to present the new doctor with the degree. You've heard the decision of the Academic Board of Wageningen University to confer on you, Theodoros A.

Golramudito, the degree of Doctor. It's now my honor to present you with a degree, signed by the Deputy Director Magnificus, the promoters and the co-promoter, and seals with the great seal of Wageningen University. I first invite you to sign the degree as well.

This is signature you declared to act according to the Netherlands Code of Conduct for Research Integrity and in the future. Allow me, Madame Director, to offer my congratulations and to add to personal events. Dear Dr.

Provenito, dear Theo. It's my pleasure to be the first to congratulate you and also on behalf of promoter Proveni-Schmidt and your co-promoter Cynthia Klosman, with obtaining the degree of Doctor, who create achievement after so many years of hard work. I would like to extend my congratulations to your mother, cousin and family present here today in the audience, your friends and also to your father and other family watching through internet. Your research was done within the Indonesian education, a research program of the Center of Higher Education Funding and assessment of the Republic of Indonesia. In Wageningen, you investigated the last four years, whether we can improve the health-promoting properties of the traditional food product, Tempe. Based on prior research, you performed in Indonesia's teacher-researcher at the Faculty of Biotechnology of the APA, Yaya, the Catholic University of Indonesia. You investigated whether you could use lactic acid bacteria to produce extra-polysaccharide unit fermentation of soybeans. This could inhibit inter-toxicgenic Asheria choline adhesion to the mammalian intestinal surface, mitigating diarrhea. You only had to screen 10 different LMB species for the APS production before you could already select four promising species to produce larger quantities of the APS. And to study those in much more detail. You discovered that the four lactic acid bacteria produced mainly two different classes of extra-polysaccharide, each with a different sub-structure and different ratio. You also discovered, explored, and validated various binding assays to estimate the highest binding capacity towards ATEC, and, or, estimating the lowest binding of ATEC to APA cells. Everything with only one purpose, creating Tempe, is enhanced by activity due to the presence of APS. Your work will result in four scientific papers, which three have been published already in high-impact journals in the field, and one paper is on the left view. It's nice to mention maybe that your first paper has already been cited for 15 times, so that's quite nice, I hope. Your thesis can be considered as a valuable piece of research, helping us not only to understand the role of lactic acid bacteria, APS, in Tempe, binding to human and animal intestines, but also to shine light on the structure-function relationship of these APSes. However, more importantly, for me, for us, I think, you clearly demonstrated that LAB fortification works in real food made through the fermentation process. Tails, some more personal words.

You really deserve the toxic title, and not only because of the nice research we're discussing today, also, and maybe even especially because of your presence to get a PhD title. After your food technology study in Wageningen, and having a position as research in Indonesia, you really decided to go for a PhD degree in the Netherlands, in Wageningen, at food chemistry, wherever. I lost counting the number of applications from you for food chemistry, PhD vacancies, because we had to disappoint you several times. But your dedication and almost stubbornness driving you to apply again and again made it hard for me and my colleagues to disagree. But

I think we both agreed today that it was the best fit between your thesis topic and your PhD track. It was a colder decision to wait a bit longer and then have the study as presented. Although you are very broadly interested, and you could have nicely many different other research projects, the research discussed today really fitted you as clothes, and you in fact became Mr.

Tempe. From the start, you knew already quite strongly what you wanted to achieve and what to investigate. You wrote quite independently a convincing grant application on your topic, and once started, you wrote your own detailed PhD research proposal. During the run of the PhD adventure, you continued in a highly driven, always nice and convincing way to focus on scientific ways to prove, to understand, your electric acid bacteria, the APS, input application, and to bring it into practice. Upon the start of your thesis research, you brought in already quite some knowledge, know-how and hands-on from your research in Indonesia. This is certainly true for the microbial and tempe-making part of your research while you felt less secure on the chemistry and the characterization of the APS. As demonstrated in your thesis, page minus five, I will not ask one of the pioneers to read it. You stated, to those who are fighting silent battles, be kind to yourself and remember that you are not alone, you are much stronger than you think. This statement, so much applies to you, is you. Feeling sometimes uncomfortable, but always looking, learning, developing, even outside your comfort zone. You really developed yourself to become an all-around independent and sound researcher. You are curious and always willing to invest time in learning something new. In our progress meetings, we often ask you to do some more experiments or to do some further analysis to show or prove your findings. And often you reply quite enthusiastically, I have doubt already. And then it was followed by some nice results of graphs. Your way of sharing your results during presentations always contains an element of fun. First, introducing nicely the undesired traveling diarrhea by nice cartoons. Continuing with the prevention using your twin lacretic acid bacteria, APS is all explained by funny and clear graphics. Your way of interacting with people all also helped you a lot with your kind and almost innocent approach to ask for help, assistance, advice. You opened many doors and easily experienced people to very friendly, collaborative and helpful. As a second nature, you show your appreciation to all your colleagues by bringing note and unknown sweet from Indonesia and all other countries you visited. Theo, you were always around when there was something to celebrate, to party, to share, both at food chemistry as well as food microbiology. We could always hear your voice and laughter.

Also, I certainly should not complain. You played a key role in the organization of the PEC trip to Indonesia, putting the almost entire program together due to all your contacts on Java. In Indonesia, you showed your colleagues around and managed to contact with the bus drivers and all others. We heard it almost felt like minions following Theo. Theo's supervisors admired the way you integrated simultaneously in two share groups. It's not always easy. Expanding your network of people at work to an impressive size. You are certainly not yet bored nor finished in your search for healthy bacteria, inspiring and highly complex APS's. And so you would like to study further. You are still employed at ALMA-YAYA Catholic University in Indonesia, where you will continue your career in about four weeks. We all wish you a wonderful time today, this week, coming holiday, and a bright future. I am convinced that we will continue to see each other in the future and maybe even collaborate. And hereby, I give the words back to Bandhavanek. Thank you.

Well, after these nice words of your supervisors, it's now my turn to congratulate you with a yes-to-receive degree of Doctor on behalf of Wageningen University, but also on behalf of myself. I extend my congratulations to your supervising team, Dr. Scholz, Professor Scholz, Professor Scholz, Professor Schmitt and Dr. Glausterman. I also include in my congratulations your parents, other family members, friends

and colleagues present here in person or following us online. For me, one of the interesting aspects of serving as a chair for PhD defenses is that one is informed about the many different topics being studied here at Wageningen University. Today, the focus was on antimicrobial properties of tempi, originally an important dietary component in Asian food. And I read in the newspaper this week that in Jakarta now, there are 48 million people. So controlling intestinal microbes infections appears extremely important there. And even better, if that can be done by choosing the right food components. So the outcome of your PhD research received compliments from the evaluators. And I'm sure it will also give you an excellent start in your follow-up career. I wish you all the best for your future career, but also for your personal life. And finally, I'd like to thank the four examiners, Professor Scholz from Wageningen University, Professor Weijfus from Germany, Dr.

Raubus from DSM-Firmenich and  Kriels from InnoGI Technology in Delft. Thank you so much for evaluating the thesis and the performance of the candidates and to shape the scientific discussion today. Your expert help is really important for Wageningen University to keep up the standard regarding PhD degrees. It's highly appreciated. So thank you. And finally, I wish you all, and especially the young doctor, a very pleasant continuation of the day. And here it is, I close the ceremony.